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(54) Title: COMPOSITION AND METHOD FOR MODULATING DENDRITIC CELL-T CELL INTERACTION		
(57) Abstract		
<p>The present invention relates to the use of a compound that binds to a C-type lectin on the surface of a dendritic cell, in the preparation of a composition for modulating, in particular reducing, the immune response in an animal, in particular a human or another mammal. The composition in particular modulates the interactions between a dendritic cell and a T-cell, more specifically between a C-type lectin on the surface of a dendritic cell and an ICAM receptor on the surface of a T-cell. The compositions can be used for preventing/inhibiting immune responses to specific antigens, for inducing tolerance, for immunotherapy, for immunosuppression, for the treatment of auto-immune diseases, the treatment of allergy, and/or for inhibiting HIV infection. The compound that binds to a C-type lectin is preferably chosen from mannose, fucose, plant lectins, antibiotics, sugars, proteins or antibodies against C-type lectins. The invention also relates to such antibodies, and to a method for isolating dendritic cells using such antibodies.</p>		

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Titel: Composition and method for modulating dendritic cell-T cell interaction.

5 The present invention relates to compositions and a method for modulating, in particular increasing or reducing, the immune response in an animal, such as a human or another mammal.

In one embodiment, the invention relates to compositions and a method for modulating, and in particular reducing, the adhesion of dendritic cells to T cells.

10 More specifically, this embodiment of the invention relates to compositions and a method for modulating, and in particular reducing, the adhesion of C-type lectin receptors on the surface of dendritic cells to the ICAM-receptors on the surface of T cells. By modulating this adhesion, both dendritic cell-T cell interactions, such as cluster formation and antigen presentation, as well as for instance primary T cell responses dependant thereon, can be influenced,  
15 resulting in a modulation of the immune response.

The compositions and method of the invention can therefore be used to alter immune responses to specific antigens as well as immune responses caused by disorders of the immune system, such as may occur in auto-immune diseases or in allergy.

20 In a further embodiment, the method of the invention can further be used in the treatment of HIV-infections and similar disorders of the immune system, as well as to modulate the immune response to grafts or after transplant.

25 In another embodiment, the invention relates to compounds, compositions and methods for modulating, and in particular increasing, the immune response in an animal, such as a human or another mammal.

30 More specifically, in this embodiment, an immune response against a specific antigen is generated, increased or promoted by presenting said antigen or an antigenic part thereof to dendritic cells in a form that can bind to the C-type lectin receptors on the surface of dendritic cells. The antigen presented in this manner enters the dendritic cell, which in turn presents the antigen to the T-cells, thereby causing an immune response, or an increased immune response, against

the antigen.

Further embodiments of the invention relate to prophylactic techniques as well as diagnostic techniques using the compositions and/or embodying the methods as described above.

5           The invention is based on the surprising discovery that the adhesion of dendritic cells to T cells is mediated by a C-type lectin receptor on the surface of the dendritic cells. It has also been found that this C-type lectin binds to the ICAM receptors on the surface of T cells. With the term "ICAM receptor(s)" both the ICAM-2 and ICAM-3 receptor are meant, and in particular the ICAM-3  
10           receptor.

          The invention is further based on the finding that the inhibition of this C-type lectin receptor on the dendritic cells, such as by known inhibitors of C-type lectins and/or by specific antibodies directed against the C-type lectin receptor, can modulate, and more specifically reduce, the adhesion of T cells to dendritic cells,  
15           and can thereby influence the immune response, in particular the initial stages of the immune response.

          WO 96/23882 describes a murine and human receptor with C-type lectins domains that is abundantly expressed on the surface of dendritic cells and thymic epithelial cells. The murine receptor -named "DEC-205"- is described as a 205  
20           kDa protein with an isoelectric point of about 7,5 that contains 10 C-type lectin domains and that is homologous to the macrophage mannose receptor (MMR).

          WO 96/23882 further describes monoclonal and polyclonal antibodies against DEC-205. However, these antibodies were not able to block dendritic cell function. In particular, monoclonal and polyclonal anti-DEC-205 antibodies were  
25           unable to inhibit the interaction between dendritic cells and helper T cells, both *in vitro* (as determined by the inability of anti-DEC-205 to inhibit allogenic T cell proliferation in a one way mixed leucocyte reaction) and *in vivo* ( as determined by the inability of anti-DEC-205 to inhibit an *in vivo* response, i.e. in a local graft-versus-host (GVH) reaction). These results suggest that the DEC-205 receptor  
30           is not involved in dendritic cell-T cell interaction (i.e. adhesion) and that the anti-DEC-205 antibodies cannot be used to modulate the immune response.

Curtis et al., in *Proc.Natl.Acad.Sci. USA*, 89 (1992), p. 8356-8360, as well as in WO 93/01820, describe a non-CD4 gp120 receptor isolated and cloned from human placenta tissue. This gp120 receptor is expressed on mammalian cells which do not exhibit high levels of CD4, such as placenta, skeleton muscle, brain, neural and mucosal cells, as well as other tissues and cells including colon, thymus, heart, T cells, B cells and macrophages ( but not in the liver or the kidney). The amino acid sequence of the C-type lectin gp120 receptor disclosed in SEQ ID's no. 1 and 2 of WO 93/01820 has a high degree of sequence homology (>98%) with the C-type lectins that are now found to be present on dendritic cells.

Curtis and WO 93/01820 further discuss the role this C-type lectin receptor plays in the infection of the aforementioned cells/tissues with HIV, i.e. by binding the major HIV envelope glycoprotein gp120 prior to internalization of the virion into the cell. It was found that inhibition of the C-type lectin gp120 receptor can reduce or inhibit HIV infection of these cells/tissues. As suitable inhibitors, WO 93/01820 discloses mannose carbohydrates, fucose carbohydrates, plant lectins such as concanavalin A, specific antibiotics such as pradimicin A, and sugars such as N-acetyl-D-glucosamine and galactose ( which however are described as less potent). These compounds and compositions containing them are used either *in vitro* or *in vivo* to inhibit the binding of HIV to the cell surface.

WO 93/01820 further discloses that binding of HIV to COS-7 cells can be inhibited by pre-incubation of gp120 with an anti-gp120 monoclonal antibody (named "antibody 110.1"). However, this antibody is not directed against the C-type lectins, but against the gp120 protein.

However, neither Curtis nor WO 93/01820 mentions or suggests the presence of such a C-type lectin on dendritic cells, nor do these references mention or suggest their role in dendritic cell - T cell interaction during the initial stages of an immune response.

WO 95/32734 describes FcγRII (CD32) bridging (or crosslinking) compositions and their use in modulating the immune response to specific antigens. This reference is based upon the finding that the bridging of FcγRII (CD32) molecules on antigen presenting cells (APCs) impaires the expression of

the essential co-stimulatory molecules B7-1/2 (i.e. prevents their up-regulation) and causes thereby impairs the expression of (i.e. causes the down-modulation of) the adhesion molecule ICAM-3, with the functional consequence of an impaired capacity of the monocytes to co-stimulate the activation of antigen-specific T cells (i.e. resulting in the modulation of antigen-specific T cell unresponsiveness). The bridging agent is chosen from aggregated human IgG molecules or Fc-fragments thereof; bi- or multivalent monoclonal antibodies to FcγRII or fragments thereof, or a fusion of two or more humane IgG Fc parts.

WO 95/32734 is therefore directed towards modulating (i.e. inhibiting) the co-stimulation signal required for T cell activation (i.e. besides the primary signal of TcR/CD3 interaction), in particular to induce proliferation and maturation into effector cells. WO 95/32734 is not directed towards modulating dendritic cell - T cell adhesion, nor does it disclose or suggest either the presence of C-type lectins on (the surface of) dendritic cells or their interaction with the ICAM-3 receptors on T cells.

WO 98/02456 discloses a group II human C-type lectin isolated from a stimulated human macrophage library. WO 98/49306 discloses a group IV C-type lectin present in human pancreatitis-associated protein ("PAP"). WO 98/41633 discloses a group V human C-type lectin isolated from a human tumor clone.

WO 98/02456, WO 98/49306 and WO 98/41633 further disclose methods for producing antibodies against these C-type lectins.

However, none of these references relates to C-type lectins on dendritic cells; the C-type lectins disclosed in these references differ from the C-type lectins described therein in origin, in biological function, and in structure.

Dendritic cells (DC) are professional antigen-presenting cells that capture antigens in the peripheral tissues and migrate via lymph or blood to the T cell area of draining lymph nodes and spleen. Here they present processed antigens to naive T cells, initiating antigen-specific primary T cell responses.

Due to their position in the body surface as immunosurveillance cells, it is likely that DC are the first cells infected with HIV-1 after mucosal exposure and are therefore implicated to play an important role in the immunopathogenesis of

HIV. It is now generally believed that HIV converts the normal trafficking process of DC to gain entry into lymph nodes and access to CD4<sup>+</sup> T cells, as was demonstrated *in vivo* using primary simian immunodeficiency virus infection of macaque as a model system (Spira et al., 1996)(Joag et al., 1997). Productive infection of DC with HIV-1 has been reported by several investigators (Granelli-Piperno et al., J Virol 72(4), 2733-7, 1998) (Blauvelt et al., Nat Med 3(12), 1369-75, 1997.) and substantial evidence indicates that DC pulsed with HIV-1 *in vitro* can induce a vigorous infection when co-cultured with T cells (Cameron et al., Science 257(5068), 383-7, 1992). Although it is still unclear whether a similar scenario occurs in HIV infected individuals, HIV-1 transmission from DC to T cells could contribute to the CD4<sup>+</sup> T cell depletion observed in AIDS. Studying HIV-DC interactions should contribute to the understanding of early events of HIV infection and will hopefully lead to strategies aimed at blocking early events in transmission. For a further discussion, reference is also made to WO 95/32734 and WO 96/23882.

DC are unique in their ability to interact with and activate resting T cells. However, prior to the present invention, it was largely unknown how DC-T cell contact is initiated and regulated. Herein, the role of ICAM-3 in DC-T cell interactions is investigated. It is demonstrated that although DC strongly adhere to ICAM-3, this adhesion is not mediated by LFA-1,  $\alpha$ D or any other integrin. In the search for this novel ICAM-3 receptor on DC a C-type lectin receptor was cloned, designated DC-SIGN, that is preferentially expressed by DC. Besides its prominent role in DC-T cell clustering and initiation of T cell responses we discovered that DC-SIGN is a major HIV-1 receptor involved in infection of DC and subsequent HIV-1 transmission to T cells. Thus HIV-1 and resting T cells exploit a similar highly expressed receptor to interact with DC.

In a first aspect, the invention relates to the use of a compound that binds or can bind to a C-type lectin on the surface of a dendritic cell, in the preparation of a composition for modulating, in particular reducing, the immune response in a animal, in particular a human or another mammal.

In particular, this aspect of the invention relates to the use of a compound

that binds or can bind to a C-type lectin on the surface of a dendritic cell, in the preparation of a composition for modulating, in particular inhibiting, the interaction(s) between a dendritic cell and a T cell.

5 More in particular, this aspect of the invention relates to the use of a compound that binds or can bind to a C-type lectin on the surface of a dendritic cell in the preparation of a composition for modulating, in particular reducing, the adhesion between a dendritic cell and a T cell.

10 Especially, this aspect of the invention relates to the use of a compound that binds or can bind to a C-type lectin on the surface of a dendritic cell in the preparation of a composition for modulating, in particular reducing, the adhesion between a C-type lectin on the surface of a dendritic cell and an ICAM receptor on the surface of a T cell.

15 DC-SIGN — The amino acid sequence of one C-type lectin that was found to be involved in the binding of the dendritic cells to the T-cells is shown in SEQ ID no. 1 and Figure 9. This C-type lection receptor is essentially similar to to the C-type lectin gp120 receptor described by Curtis et al. in *Proc.Natl.Acad.Sci. USA*, 89 (1992), p. 8356-8360 and in WO 93/01820. In particular, it has a high degree of homology (< 98%) to the amino acid sequence given in SEQ ID no.1 of WO 93/01820. It is a group II C-type lectin of 404 amino acids; with an apparant Mr of about 44 kDa; and with a first domain (Met 1 to Ala 76) comprising a cytoplasmic amino terminus, a second domain (Ile 77 to Val 249) comprising tandem repeats, and a third domain (Cys 253 to Ala 404) with a high degree of homology to other known C-type lectins which are type II membrane proteins. Further characterisation is given below.

20 In the invention, this C-type lectin on dendritic cells was found to bind to ICAM receptors on the surface of T-cells.

Accordingly, the compositions of the invention can be used to modulate (i.e. alter and/or modify), and more specifically inhibit (i.e. reduce and/or down-tune), the interaction(s) between dendritic cells and T cells.

30 Such interactions include the adhesion of T-cells to dendritic cells, for instance in dendritic cell - T cell clustering, T-cell activation and further include

all interactions that rely on contact between dendritic cells and T-cells, by which is meant either direct cell-to-cell contact or close proximity of dendritic cells and T cells.

5 Such further interactions include, but are not limited to, processes involved in generating an immune response, in particular during the initial stages of such a response, such as primary sensitisation/activation of T-lymphocytes, (i.e. presentation of antigen and/or MHC-bound peptides to T-cells) and co-stimulation of T cells; as well as processes such as chemical signalling, endocytosis and trans-epithelial transport. For a discussion of dendritic cell-T cell interactions in general,  
10 all of which may be influenced by the compositions of the invention, reference is made to the discussion below as well as to WO 95/32734 and WO 96/23882.

The compositions of the invention can therefore be used to influence the immunomodulatory ability of dendritic cells; to modulate, and in particular reduce, dendritic cell-mediated (primary) T cell responses, and/or generally to influence,  
15 and in particular inhibit, the immune system.

Some specific applications include preventing or inhibiting immune responses to specific antigens; inducing tolerance; immunotherapy; immunosuppression, for instance to prevent transplant rejection; the treatment of auto-immune diseases such as thyroiditis, rheumatoid arthritis, systemic lupus  
20 erythematosus (SLE), multiple sclerosis and auto-immune diabetes; and the prevention or treatment of allergies.

The compositions of the invention may also modulate the activation of other receptors on T cells which are dependant upon the adhesion or close proximity of dendritic cells to T cells. Furthermore, the finding of the invention  
25 that a C-type lectin on dendritic cells binds to the ICAM receptors on T cells may open up new strategies or possibilities for influencing the interaction between dendritic cells and T cells, and thereby for modulating the immune system in general.

Furthermore, the compositions of the invention can be used to prevent or  
30 reduce the transfer of matter from dendritic cells to T cells, such as chemicals, signalling factors such as chemokines and/or interleukines, etc., and in particular

of viral particles such as HIV. In this way, by using the compositions of the invention, not only can the initial adhesion of HIV to dendritic cells be inhibited, but also the spread of HIV infection from dendritic cells to T cells.

5 This finding is of particular importance as it is thought that dendritic cells may serve as a "reservoir" of HIV, in particular during the initial stages of HIV infection. The compositions of the invention can therefore not only be used to prevent HIV infection of dendritic cells, but also to reduce the spread of HIV infection to T cells after the dendritic cells have been infected, thereby slowing down the disease process.

10 Also, it is known that activation of T cells -i.e. in the lymph glands- plays an important role in the development of AIDS in a HIV-infected patient. It is believed that the compositions of the invention may be used to prevent, inhibit or at least delay said T-cell activation and thereby slow the onset and/or the progress of the disease.

15 Therefore, in a further aspect, the invention further relates to the use of a compound that binds or can bind to a C-type lectin on the surface of a dendritic cell, in the preparation of a composition for inhibiting the HIV infection of dendritic cells, in particular for inhibiting the adhesion of HIV surface protein (i.e. gp120) to the surface of a dendritic cell and thereby the entry of HIV into said  
20 dendritic cell.

The invention further relates to the use of a compound that binds or can bind to a C-type lectin on the surface of a dendritic cell, in the preparation of a composition for inhibiting the transfer of HIV from infected dendritic cells to non-infected T cells.

25 Compounds that can be used in the compositions of the invention include inhibitors for the C-type lectins known per se, including but not limited to those described in WO 93/01820 as mentioned above.

In general, these are compounds that can bind or adhere to (preferably in a reversible manner), or that can serve as a ligand for, the C-type lectins, in  
30 particular the C-type lectin of SEQ ID no.1/Figure 9 or natural variants or equivalents thereof. Examples are mannose carbohydrates such as mannan and D-

mannose; fucose carbohydrates such as L-fucose; plant lectins such as concanavalin A; antibiotics such as pradimicin A; sugars such as N-acetyl-D-glucosamine and galactose ( which however are described as less potent); as well as suitable peptidomimetic compounds and small drug molecules, which can for instance be identified using phage display techniques. Furthermore, proteins such as gp120 and analogs or fragments thereof or similar proteins with binding capacity to C-type lectins on dendritic cells may be used, as well as isolated ICAM-receptors and analogs thereof, including part or fragments thereof. Such parts or fragments should then preferably still be such that they can bind to the C-type lectins on the surface of dendritic cells.

However, the use of carbohydrates is usually less desired from a therapeutic point of view, as such they can be rapidly metabolized *in vivo*. Also, the use of plant lectins such as concanavalin A and pradimicin antibiotics can have disadvantages in a therapeutic setting, in particular when treating patients with auto-immune disorders and/or HIV-infections.

Preferably, one or more physiological tolerable and/or pharmaceutically acceptable compounds are used, such as defined in WO 93/01820. For instance, the use of gp120 or derivatives thereof may cause undesired side effects, in particular on the nervous system (vide WO 93/01820).

Therefore, according to the invention, preferably an antibody directed against a C-type lectins as present/expressed on the surface of a dendritic cell, or a part, fragment or epitope thereof, is used. As used herein, the term antibodies includes *inter alia* polyclonal, monoclonal, chimeric and single chain antibodies, as well as fragments (Fab, Fv, Fa) and an Fab expression library. Furthermore, "humanized" antibodies may be used, for instance as described WO 98/49306.

Such antibodies against the C-type lectins of the invention can be obtained as described hereinbelow or in any other manner known per se, such as those described in WO 95/32734, WO 96/23882, WO 98/02456, WO 98/41633 and/or WO 98/49306.

For instance, polyclonal antibodies can be obtained by immunizing a suitable host such as a goat, rabbit, sheep, rat, pig or mouse with a C-type lectin

or an immunogenic portion, fragment or fusion thereof, optionally with the use of an immunogenic carrier (such as bovine serum albumin or keyhole limpet hemocyanin) and/or an adjuvant such as Freund's, saponin, ISCOM's, aluminium hydroxide or a similar mineral gel, or keyhole limpet hemocyanin or a similar surface active substance. After an immuneresponse against the C-type lectins has been raised (usually within 1-7 days), the antibodies can be isolated from blood or serum taken from the immunized animal in a manner known per se, which optionally may involve a step of screening for an antibody with desired properties (i.e. specificity) using known immunoassay techniques, for which reference is again made to for instance WO 96/23882.

Monoclonals may be produced using continuous cell lines in culture, including hybridoma and similar techniques, again essentially as described in the above cited references.

Fab-fragments such as  $F(ab)_2$ , Fab' and Fab fragments may be obtained by digestion of an antibody with pepsin or another protease, reducing disulfide-linkages and treatment with papain and a reducing agent, respectively. Fab-expression libraries may for instance be obtained by the method of Huse et al., 1989, Science 245:1275-1281.

Preferably, a monoclonal antibody against the C-type lectin(s) on dendritic cells is used, more specifically against the peptide with the amino acid sequence shown in/encoded for by SEQ ID no's 1 and 2 and Figure 9, or (an antigenic) part thereof; and such monoclonals are a further aspect of the invention. Hereinbelow, the invention will be illustrated by means of two such monoclonals, herein referred to as AZN-D1 and AZN-D2, although similar monoclonals with comparable specificity for C-type lectins may also be used.

In a further aspect, the invention provides a cell line such as a hybridoma that produces antibodies, preferably monoclonal antibodies, against the C-type lectins on dendritic cells, more specifically against the peptide with the amino acid sequence shown in/encoded for by SEQ ID no's 1 and 2 and Figure 9 or (an antigenic) part thereof. Hybridomas that produce the abovementioned monoclonals AZN-1 and AZN-2 of the invention were deposited on April 8, 1999 with the

European Collection of Cell Cultures under (provisional) ECACC accession numbers 990400818 and 99040819, respectively.

The invention also relates to a method for producing an antibody, preferably a monoclonal antibody, against the C-type lectins on dendritic cells, more specifically against the peptide with the amino acid sequence shown in ( or  
5 encoded for) by SEQ ID no's 1 and 2 and Figure 9 or (an antigenic) part thereof, said method comprising cultivating a cell or a cell line that produces said antibody and harvesting/isolating the antibody from the cell culture.

Neither (monoclonal) antibodies against the C-type lectins on dendritic  
10 cells, nor cells or cell lines that produce such antibodies, have to date been described in the art, and it is envisaged that the novel antibodies of the invention will have broad applicability (i.e. besides the pharmaceutical/therapeutic uses disclosed herein). Some of these application -which form yet another aspect of the invention- will be clear to the skilled person from the disclosure herein.

For instance, the antibodies of the invention can be used to detect the  
15 presence of (and thereby determine the expression of) C-type lectins in or on tissues or whole cells, as well as the detect the presence of C-type lectins in other biological samples such as cell fragments or in cell preparations. The information thus obtained can then (also) be used to determine whether the method or  
20 compositions of the invention can be applied to such tissues or cells. The antibodies of the invention could also be used to detect (qualitatively and/or quantitatively), isolate, purify and/or produce dendritic cells, for instance in/from biological samples, including biological fluids such as blood, plasma or lymph fluid; tissue samples or cell samples such as bone marrow, skin tissue, tumor tissues, etc; or  
25 cell cultures or cultivating media.

For instance, the few methods presently available for isolating/producing dendritic cells from biological samples - such as the method described in US-A-  
5,643,786, comprising leukapheresis followed by fluorescence-activated cell-sorting - are very cumbersome multi-step procedures that provide only low yields and  
30 heterogenous samples. As a result, the limited availability of dendritic cells has severely hindered research into this important class of cells.

By using the monoclonals of the invention, dendritic cells could be isolated and produced with high(er) yield and with high specificity. In such a method, the monoclonals could be used in a manner known per se for the harvesting, isolation and/or purification of cells from biological fluids using antibodies.

5 For instance, the monoclonals could be attached to a column or matrix, to (para)magnetic beads or to a similar solid support, which could then be contacted with a biological sample or culture medium containing dendritic cells. The cells that do not attach themselves to the carrier are then separated or removed -e.g. by washing- after which the dendritic cells are separated from the carrier and isolated  
10 in a manner known per se.

Also, the monoclonals of the invention could be used to detect/determine the presence of dendritic cells (and/or C-type lectins) and/or the expression of genes coding therefor in biological samples, in which the antibodies could again be used in a manner known per se for the analytical of antibodies, such as  
15 competitive inhibition assays or ELISA-type immunoassays. For instance, the antibodies could be used in combination with microscopy techniques, cell sorting techniques including flow-cytometry and FACS, techniques based upon solid supports and/or detectable labels or markers (which can be attached to the antibodies), techniques baed upon (para)magnetic beads or any other detection or  
20 assay technique known per se in which antibodies can be used. Such assays and kits for therein - which besides the antibodies of the invention can contain all further components known per se for antibody-based assays, as well as manuals etc.- form a further aspect of the invention.

Thus, the monoclonals of the invention constitute a very useful diagnostic  
25 and research tool, for use both *in vitro* as well as *in vivo*. Possible non-limiting fields of application include the study of dendritic cells and their function and interactions; the study of the immune system; the detection of dendritic cells and/or C-type lectins in cells, tissues or biological fluids such as synovial tissue and skin tissue/skin cells ( dermal dendritic cells); as well as the study of the role  
30 denditric cells play in biological processes or disease mechanisms, such as cancer ( as dendritic cells are exploited *in vivo* in clinical trials to irradiate tumor

formation and development) and auto-immune diseases (including for instance rheumatoid arthritis).

For a further description of the methods and techniques known per se in which the antibodies of the invention can be used, reference is made to the general textbooks, such as D.P. Sites, A.I. Terr, T.G. Parslow: "Basic and clinical immunology", 8th Ed., Prentice-Hall (1994); I. Roitt, J. Brostoff, D. Male: "Immunology", 2nd. Ed., Churchill Livingstone (1994); all incorporated herein by reference. Particular reference is made to the general uses of antibodies and techniques involved therein as mentioned in sections 2.7 to 2.17 of the general reference work by Janeway-Travers: "Immunobiology, the immune system in health and disease", Third Edition.

A composition of the invention may contain two or more of the above-mentioned active compounds, or such compounds may be used in combination. For instance, an anti-C-type lectin antibody can be formulated with mannose or fucose carbohydrates, lectins and/or antibiotics such as pridamycin A, whereby a synergistic effect may be obtained.

The compositions of the invention may also contain or be used in combination with known co-inhibitory compounds, such as anti-LF3A; as well as other active principles known per se, depending upon the condition to be treated. For instance, the compositions of the invention may be formulated or used in combination with immunosuppressants (i.e. for preventing transplant rejection), immunomodulants, antibiotics, auto-antigens or allergens (for instance as described in WO 95/3234 or WO 96/23882), Tumor Necrosis Factor (TNF), and anti-viral agents such as anti-HIV agents and CD4 inhibitors including CD4 directed antibodies such as Leu-3A, whereby again a synergistic effect can be obtained.

The compositions of the invention can further be formulated using known carriers and/or adjuvantia to provide a pharmaceutical form known per se, such as a tablet, capsule, powder, freeze dried preparation, solution for injection, etc., preferably in a unit dosage form. Such pharmaceutical forms, their use and administration (single or multi dosage form), as well as carriers, excipients, adjuvantia and/or formulants for use therein, are generally known in the art and

are for instance described in WO 93/01820, WO 95/32734, WO 96/23882, WO 98/02456, WO98/41633 and/or WO 98/49306, all incorporated herein by reference. Furthermore, the formulation can be in the form of a liposome, as described in WO 93/01820.

5           Pharmaceutical formulations of antibodies, their administration and use, are generally described in WO 95/32734, WO 96/23882, WO 98/02456, WO 98/41633 and/or WO 98/49306

          The compositions of the invention may further be packaged, for instance in vials, bottles, sachets, blisters, etc.; optionally with relevant patient information  
10           leaflets and/or instructions for use.

          In a further aspect the invention relates to a method for modulating the immune response in an animal, in particular a human or another mammal, comprising administering to said animal a compound that binds or can bind to a C-type lectin on the surface of a dendritic cell, preferably in the form of a  
15           composition as described above, in an amount sufficient to alter or modify an immune response.

          The compound or composition is in particular administered in such an amount that the interaction(s) between dendritic cells and T cells are altered or modified, more in particular in such an amount that the adhesion of dendritic cells  
20           to T cells is reduced.

          This method can be used for preventing and/or treating disorders of the immune system, as well as to prevent transplant rejection, as described above.

          The invention further relates to a method for the prevention or treatment of HIV infections, comprising administering to a HIV infected patient or a person  
25           at risk of becoming HIV infected, a compound that can binds or bind to a C-type lectin on the surface of a dendritic cell, in such an amount that the adhesion of HIV to the dendritic cells, and in particular of the gp120 envelop protein of HIV to the C-type lectin on the surface of dendritic cells, is inhibited.

          Also, the invention further relates to a method for the treatment of HIV  
30           infections, comprising administering to a HIV infected patient a compound that binds or can bind to a C-type lectin on the surface of a dendritic cell, in such an

amount that the transfer of HIV from infected dendritic cells to non-infected T cells is inhibited.

5 In a further aspect, the invention is used to modulate, and in particular generate, increase and/or promote, an immune response in an animal, such as a human or another mammal, against a specific antigen or combination of antigens, by presenting said antigen(s) or one or more antigenic parts thereof to dendritic cells in a form that can bind to the C-type lectin receptors on the surface of dendritic cells. The antigen(s) presented in this manner enter(s) the dendritic cell, which then in turn presents the antigen to the T-cells, thereby causing an immune response against the antigen(s).

10 With "a form that can bind to the C-type lectin receptors on the surface of dendritic cells" is generally meant that the antigen or antigenic fragment is attached to a compound, ligand or residu that can bind to a C-type lectin on the surface of a dendritic cell, such as the compounds/ligands mentioned above or a part thereof. Said attachment can for instance be by (preferably covalent) binding, ligand-ligand interaction, complexing, ligation, fusion of proteins (e.g. through expression of said fusions), or by any other type of physical or chemical interaction or bond that enables the antigen to be presented to a dendritic cell in conjunction with the ligand for the C-type lectin, i.e. combined into a stable or semi-stable entity.

20 For instance, the antigen can be provided with the abovementioned mannose and fucose carbohydrates as covalently bound groups or side-chains; can be covalently attached to plant lectins such as concanavalin A or antibiotics such as pradimicin A; or can be provided with sugar residues such as N-acetyl-D-glucosamine and galactose (which however is less preferred), all of which serve to "direct" the antigen to the dendritic cell.

25 Preferably, the antigen is attached to (e.g. fused with or covalently bonded to) a protein that can bind to or serve as a ligand for the C-type lectins, such as gp120 and analogs thereof or the ICAM-receptors and analogs thereof, or to a part of fragment of such a protein. Alternatively, the antigen can be attached to (e.g. fused with or covalently bonded to) an antibody directed against the C-

type lectins, preferably a monoclonal antibody such as AZN-D1 and AZN-D2 mentioned above; or to a part or fragment of such an antibody as described above.

The antigen can be any antigen against which an (increased) immune response is to be obtained, or any part or fragment thereof. Preferably, any such part of fragment is such that it *per se* is capable of eliciting an immune response, such as an epitope. However, this is not required: because according to the invention the fragments are directed to the dendritic cells, i.e. with increased specificity or affinity, part fragments that would normally be incapable of eliciting an immune response may provide an immune response when used in conjunction with a ligand for the C-type lectins as described herein. Also, in general, using an antigen in combination with a ligand for the C-type lectins may increase the potency of the antigen, i.e. provide a higher or stronger immune response per unit of antigen administered. In this way, antigens - including those present in serums or vaccines, but also retroviral vectors encoding a desired antigen - could be administered at a lower dosage and still provide sufficient immune response.

Examples of suitable antigens are cancer antigens including gp 100, g250, p53, MAGE, BAGE, GAGE, MART 1, Tyrosinase related protein II and Tyrosinase related protein; all of which can be used to generate an immune response against the tumor cells that contain or express said antigen. Other types of antigen that can be used in the invention include essentially all antigens used in vaccines against (infectious) diseases, such as influenza, mumps, measles, rubella, diphtheria, tetanus, diseases due to infection with micro-organisms such as *Haemophilus influenzae* (e.g. type b), *Neisseria*, *Bordetella pertussis*, *Polyomyelitus*, Influenza virus and *Pneumococcus*, and generally any other infection or disease against which a vaccine can be developed or can be envisaged, including also parasitical, protozoan and/or viral infections such as HIV and herpes. To provide serums or vaccines, the compounds of the invention may further be combined with other antigens known *per se*.

This aspect of the invention therefore relates to the use of a combination of: 1) a compound that binds to a C-type lectin on the surface of a dendritic cell; and attached thereto: 2) an antigen or a fragment or part thereof; in the preparation

of a composition for modulating, in particular generating, increasing and/or promoting, an immune response in a animal, in particular a human or another mammal, against said antigen.

5 These combinations (e.g. in the form of a complex, a chemical substance or entity, or a fused protein or protein structure), which as such form another aspect of the invention, can again be formulated and administered in a manner known per se, such as described above.

10 In all the above methods en embodiments, the compounds/compositions used will be administered in a therapeutically effective amount, for which term reference is generally made to WO 93/01820, WO 95/32734 and/or WO 96/23882. The administration can be a single dose, but is preferably part of a multidose administration regimen carried out over one or more days, weeks or months.

15 All terms used herein have the normal meaning in the art, for which reference can be made to *inter alia* the definitions given in WO 93/01820, WO 95/32734, WO 96/23882, WO 98/02456, WO98/41633 and/or WO 98/49306, analogously applied.

20 Furthermore, although the invention is described herein with respect to the specific 44kDa C-type lectin receptor disclosed herein, it is not excluded that other, generally similar C-type lectins, including natural variants of the sequence of SEQ ID no.1 and Figure 9, may also be present on dendritic cells and/or may be involved in dendritic cell - T cell interaction. Such variants will usually have a high degree of amino acid homology (more than 80% to more than 90%) with, and/or be functionally equivalent to the specific C-type lectin disclosed herein. Also, any such receptor will generally display properties similar to those as  
25 described herein; in particular that inhibition of this receptor, either by carbohydrate inhibitors or specific antibodies, will lead to an alteration of dendritic cell/T-cell interaction. Any such variant receptors should however be distinguished from the C-type lectin receptor disclosed in WO 96/23882, inhibition of which does not result in inhibition of the interaction of dendritic cells and T-cells.

30 The invention will now be further illustrated by means of the Experimental Part given hereinbelow, as well as the Figures, in which:

- Figures 1A - 1C are graphs showing: spontaneous adhesion of leukocytes to ICAM-1 and ICAM-3 (fig. 1A); adhesion of leukocytes to ICAM-3 after activation of  $\beta$ 2-integrins (fig. 1B); adhesion of DC to ICAM-3 in the presence of blocking antibodies (20 $\mu$ g/ml) against  $\beta$ 2-integrins (NKI-L19),  $\beta$ 1-integrin (AIIB2), ICAM-3 (CBR-IC3/1, CBR-IC3/2) or in the presence of EDTA (5mM) or EGTA (5mM) (fig. 1C).
- Figures 2A - 2C are graphs showing that the antibodies AZN-D1 and AZN-D2 inhibit adhesion of DC to ICAM-3 and recognize an antigen that is specifically expressed by DC.
- Figures 3A and 3B show that DC-SIGN is identical to human placenta HIV gp120 binding C-type lectin, as can be seen from SDS-PAGE (fig. 3A) and by schematic presentation of DC-SIGN isolated from human DC (3B).
- Figures 4A and 4B show that DC-SIGN, overexpressed in COS7 cells, is recognized by the anti-DC-SIGN antibody AZN-D1 and binds to ICAM-3.
- Figure 5 shows the tissue distribution of DC-SIGN as determined by immunohistochemical analysis of the expression of DC-SIGN in tonsils (A and B) and lymph node sections (C and D) (OMx100).
- Figures 6A - 6D show that DC-SIGN mediated adhesion of DC to ICAM-3 is involved in the DC-T-lymphocyte interaction, as demonstrated by DC-SIGN mediated adhesion of DC to ICAM-3 (fig. 6A); heterotypic cell clustering of DC with K562-ICAM-3 cells (fig. 6B); dynamic cell clustering of DC with resting PBL ( fig. 6C); and the role of DC-SIGN-ICAM-3 interaction plays in DC-induced T-cell proliferation (fig. 6D).
- Figure 7 shows that DC SIGN is a receptor for HIV-1 on DC.
- Figure 8 shows that DC SIGN binds to both ICAM-3 as well as ICAM-3 expressing K562 cells.
- Figure 9 shows the sequence of DC-SIGN.

### Experimental

Dendritic cells (DC) capture antigens and migrate to secondary lymphoid tissues where they present antigens to naive T cells. HIV-1 subverts this unique capacity to gain access to CD4<sup>+</sup> T cells. In the invention, a DC specific C-type lectin was cloned, designated DC-SIGN, that not only binds to ICAM-2 and/or ICAM-3 with high affinity but is also able to bind HIV-1. Also, anti-DC-SIGN antibodies were developed that not only inhibit transient DC-T cell interactions and DC induced T cell proliferation but also effectively inhibit HIV-1 infection of DC. These findings not only have important consequences for the understanding on CD4-independent HIV entry into DC but also shed new light on the role of DC-SIGN in initiating primary immune responses.

**Example 1: Adhesion of DC to ICAM-3 is not mediated by integrins**

The role of ICAM-3 mediated adhesion in first DC-T cell contact was investigated. Exploiting a novel flowcytometric adhesion assay involving ICAM-3-Fc chimera coated fluorescent beads (Geijtenbeek et al.), the capacity of DC, resting peripheral blood lymphocytes (PBL) and monocytes to bind to this integrin ligand was tested. Immature DC, obtained after culturing of monocytes for 7 days in the presence of IL-4 and GM-CSF, strongly bind ICAM-3 without prior activation of  $\beta 2$  integrins (72%, Figure 1A). Figure 1 demonstrates that the adhesion of DC to ICAM-3 is Ca<sup>2+</sup>-dependent and integrin-independent: in Figs. 1A, B and C one representative experiment of at least 3 is shown (SD<5%).

- 1A: Spontaneous adhesion of leukocytes to ICAM-1 and ICAM-3. Freshly isolated PBL, monocytes and DC were incubated for 30 min. at 37°C with either ICAM-1Fc or ICAM-3Fc fluorescent beads. After washing, the percentage of cells that bound beads was determined by flowcytometry.
- 1B: Adhesion of leukocytes to ICAM-3 after activation of  $\beta 2$ -integrins. Binding of fluorescent ICAM-3Fc beads was measured after 30 min. at 37°C in the presence of either PMA 980 nM) or the activating anti- $\beta 2$ -integrin antibody KIM185 (10 $\mu$ g/ml). Inhibition of the LFA-1 specific adhesion after PMA activation was determined in the presence of the blocking anti-LFA-1 antibody NKI-L15 (20 $\mu$ g/ml).

- 1C: Adhesion of DC to ICAM-3 in the presence of blocking antibodies (20 $\mu$ g/ml) against  $\beta$ 2-integrins (NKI-L19),  $\beta$ 1-integrin (AIIB2), ICAM-3 (CBR-IC3/1, CBR-IC3/2) or in the presence of EDTA (5mM) or EGTA (5mM). The adhesion was determined as described in Fig. 1A.

5 This spontaneous binding of DC to ICAM-3 is stronger than that of monocytes, whereas resting PBL hardly bind ICAM-3 (Figure 1A). Adhesion of DC to ICAM-3 could not be blocked with any anti- $\alpha$ L or anti- $\beta$ 2 integrin antibody (Figure. 1A). In contrast, adhesion of monocytes to ICAM-3 is LFA-1 dependent, since adhesion is blocked by anti- $\alpha$ L antibodies (Figure 1A). Since neither antibodies directed  
10 against the other  $\beta$ 2 integrin members ( $\alpha$ D $\beta$ 2,  $\alpha$ M $\beta$ 2,  $\alpha$ X $\beta$ 2, data not shown), nor antibodies directed against other integrins ( $\beta$ 1,  $\beta$ 7 integrins, Figure 1B), blocked the adhesion of DC to ICAM-3, it was concluded that the binding of DC to ICAM-3 is integrin-independent.

The interaction of DC with ICAM-3-Fc beads is ICAM-3 specific since  
15 the anti-ICAM-3 antibodies CBR3/1, CBR3/2 of the invention and a combination of both antibodies are able to inhibit the adhesion to a large extent (Figure 1B). Interestingly, adhesion of DC to ICAM-3 could be completely blocked by EDTA and EGTA (Figure 1B). These findings strongly suggest that DC bind ICAM-3 through a  $Ca^{2+}$  dependent surface receptor that does not belong to the  $\beta$ 1 or  $\beta$ 2  
20 integrin family. This molecule was designated: DC-Specific ICAM-3 Grabbing Non-integrin ( DC-SIGN).

#### **Example 2: Antibodies against DC-SIGN inhibit the DC-ICAM-3 interaction.**

To investigate DC-SIGN in more detail, antibodies against the ICAM-3  
25 binding receptor were raised. Spleens of mice immunized with DC were fused with SP2/0 myeloma cells and supernatants of the resulting hybridomas were screened for the presence of antibodies capable of inhibiting DC specific adhesion to ICAM-3. Two hybridomas were selected, cloned and the resulting antibodies were named AZN-D1 and AZN-D2. Both purified antibodies strongly inhibit  
30 adhesion of DC to ICAM-3, but do not affect LFA-1 mediated adhesion of monocytes to ICAM-3 (Figure 2A). Figure 2 demonstrates that antibodies AZN-

D1 and AZN-D2 inhibit adhesion of DC to ICAM-3 and recognize an antigen that is specifically expressed by DC:

- 2A: The monoclonal antibodies AZN-D1 and AZN-D2 (20 µg/ml) block adhesion of DC but not that of freshly isolated monocytes to fluorescent ICAM-3Fc beads. A representative experiment of at least 3 experiments is shown (SD<5%).

- 2B: DC-SIGN expression increased during DC development. DC were cultured from monocytes in the presence of GM-CSF and IL-4. At different timepoints the developing DC were analyzed for expression of the monocyte marker CD14,  $\beta 2$  integrin LFA-1 and DC-SIGN. Cells were gated on forward-side scatter and the mean fluorescence is shown in the top right corner of the histograms. A representative experiment out of 3 is shown.

- 2C: DC developing from monocytes, in the presence of GM-CSF and IL-4, increasingly bind to ICAM-3 in a DC-SIGN dependent manner. At different time points during culturing cells were harvested and incubated with fluorescent ICAM-3Fc beads in the presence of the blocking anti- $\beta 2$ -integrin antibody AZN-L19 or the AZN-D1 antibody (20µg/ml). Adhesion was determined as described in Fig. 1A. AZN-D2 inhibited adhesion to ICAM-3 similar to AZN-D1 (results not shown). A representative experiment out of 3 is shown (SD<5%).

- 2D: Relative contribution of  $\beta 2$ -integrins and DC-SIGN mediated adhesion to ICAM-3 by developing DC. Relative contribution is calculated from the inhibition of adhesion in the presence of AZN-D1 or AZN-L19 as described in Fig. 2C.

Using AZN-D1 antibodies in flowcytometric analyses it was demonstrated that DC-SIGN is not expressed by monocytes (Figure 2B). Cells expressing DC-SIGN can already be detected after 1 day of culture. The expression level of DC-SIGN increases during culture (Figure 2B). The expression of the monocyte marker CD14 gradually decreases during culture and at day 7 only a low CD14 expression is observed (Figure 2B). Further flowcytometric analyses demonstrated that at day

7 the cells also express high levels of MHC Class I, II, the  $\beta 2$  integrin p150,95 and CD86 (data not shown), consistent with the differentiation of monocytes into immature DC. These results demonstrate that DC-SIGN is abundantly expressed by DC at day 7, the expression level is several fold higher than that of LFA-1.

5 Simultaneously, the involvement of DC-SIGN in ICAM-3 binding during the differentiation of monocytes into immature DC was investigated (Figure 2C). At onset of the culture (day 0) binding to ICAM-3 by monocytes is completely  $\beta 2$  integrin (LFA-1) dependent, as demonstrated by inhibition of adhesion with the blocking anti- $\beta 2$  integrin antibody L19 (Figure 2C). At day 1, when low levels of  
10 DC-SIGN are expressed (Figure 2B), ICAM-3 specific adhesion depends on both  $\beta 2$  integrin (LFA-1) and DC-SIGN (Figure 2C). From day 2 to day 7 the ICAM-3-specific adhesion increases, becomes  $\beta 2$  integrin-independent (Figure 2C) and from day 2 is solely mediated by DC-SIGN, since anti-DC-SIGN block the adhesion completely. Maximum adhesion through DC-SIGN is reached at day 7  
15 (Figure 2C).

Together these results demonstrate that the increase in expression of DC-SIGN coincides with the observed increase in ICAM-3 binding (Figure 2A and B). From these findings it can be concluded that DC-SIGN, recognized by the antibodies AZN-D1 and AZN-D2, is the novel ICAM-3 binding receptor expressed by  
20 DC.

### Example 3: DC-SIGN is a 44 kDa protein.

To obtain information regarding the molecular weight of DC-SIGN DC-SIGN was immunoprecipitated from a lysate of  $^{125}\text{I}$ -surface labeled DC. Analysis  
25 by SDS-PAGE under reducing conditions revealed a single protein of 44 kDa (Figure 3A, lanes 1-2). Figure 3 demonstrates that DC-SIGN is identical to human placenta HIV gp120 binding C-type lectin:

- 3A: DC-SIGN is a 44 kDa protein. DC were surface labeled with  $\text{I}^{125}$ , lysed and DC-SIGN was immunoprecipitated with the anti-DC-SIGN antibodies  
30 AZN-D1 (lane 1), AZN-D2 (lane 2) and AZN-L19 (anti- $\beta 2$ -integrin; lane 3). The immunoprecipitates were analyzed by SDS-PAGE (5-15% gel)

followed by autoradiography. The migration of the molecular weight markers is indicated on the left. The arrows indicate the  $\alpha$ -chains of LFA-1 ( $\alpha$ L, 180 kDa), MAC-1 ( $\alpha$ M, 165 kDa) and p150,95 ( $\alpha$ X, 150 kDa), the  $\beta$ 2 integrin chain (95 kDa) and DC-SIGN (44 kDa). Similar results were obtained in 3 other experiments.

- 3B: Schematic presentation of DC-SIGN isolated from human DC. The two boxed peptides (aminoacid positions 296-306 and 187-197 of the human placenta gp120 binding C-type lectin ()) were identified by internal peptide sequencing of immunoprecipitated DC-SIGN using Edman degradation. The cDNA encoding DC-SIGN was isolated from DC. The deduced amino acid sequence is 100% identical to that of the human placenta gp120 binding C-type lectin (). The transmembrane region, the lectin domain and the seven complete and eight partial repeats (R1-R8) are indicated.

Analysis of the immunoprecipitate on a non-reducing SDS-PAGE gel shows that DC-SIGN exists as a monomer (results not shown). Furthermore, using ICAM-3-Fc coated beads also a 44 kDa protein could be extracted from the DC lysate whereas in the presence of blocking anti-DC-SIGN antibodies this protein could not be precipitated with ICAM-3-Fc coated beads (results not shown). These findings demonstrate that DC-SIGN is expressed by DC as a 44 kDa protein under reducing conditions. The observation that ICAM-3 Fc coupled beads only extracted a 44 kDa protein out of the DC lysate indicates that DC-SIGN has a high affinity for ICAM-3, much higher than LFA-1 or  $\alpha$ D $\beta$ 2 which are also expressed by DC (Figure 3A) and have been reported to bind ICAM-3 (Vandervieren et al., Immunity. 3, 683-690, 1995). Since very low amounts of LFA-1 are immunoprecipitated in comparison to DC-SIGN (Figure 3A, lane 1 and 3) this confirms that DC-SIGN is more abundantly expressed by DC than LFA-1. Together, these data demonstrates that DC-SIGN is a single polypeptide of 44 kDa and is the primary receptor for ICAM-3 on DC.

Example 4: DC-SIGN is identical to the human HIV gp120 binding C-type

lectin.

To identify DC-SIGN a preparative immunoprecipitation from a DC lysate with the anti-DC-SIGN antibody AZN-D1 and excised the 44 kDa protein from the SDS-PAGE gel was performed. After tryptic digestion, the resulting peptides were extracted from the gel and purified using preparative HPLC. Subsequently, the amino acid sequences of 2 peptides (0.5-1 pmol) were determined using the Edman degradation procedure. Both peptides consisted of 11 amino acid residues (Figure 3B) and the peptide sequences were used to screen the EMBL database for homology with known proteins. Both peptides proved 100% identical to the deduced amino acid sequence of the human HIV gp120-binding C-type lectin (Curtis et al., 1992). This protein has previously been identified exclusively in placenta as a CD4-independent receptor for the human immunodeficiency virus (HIV) envelope glycoprotein gp120.

RT-PCR analysis with primers based on the gp120-binding C-type lectin sequence, on RNA isolated from DC yielded a PCR product of the expected length of 1237 nt. The DC-specific PCR product was cloned and sequencing confirmed 100% identity with the human gp120-binding lectin (Figure 3B). Flowcytometric analysis of COS7 cells, transfected with the cDNA encoding the placenta gp120-binding C-type lectin, unequivocally proves that the gp120 binding C-type lectin is indeed identical to DC-SIGN (Figure 4A). Figure 4 demonstrates that DC-SIGN, overexpressed in COS7 cells, is recognized by the anti-DC-SIGN antibody AZN-D1 and binds ICAM-3:

- 4A: AZN-D1 recognizes COS7 cells transfected with the cDNA encoding DC-SIGN (filled) and not mock transfected COS7 cells (open). AZN-D2 gave a similar staining (results not shown).
- 4B: Adhesion of COS7-DC-SIGN to ICAM-3. COS7 cells were transfected and the adhesion was determined as described in Fig. 1A, respectively. Adhesion of COS7-DC-SIGN cells to ICAM-3 was measured in the presence of EGTA (5 mM) and blocking antibodies against DC-SIGN (AZN-D1), ICAM-3 (CBR-IC3/1, CBR-IC3/2) and  $\beta$ 2 integrins (AZN-L19). A representative experiment out of 3 is shown (SD<5%).

About 30% of the transfected COS7 cells are stained with anti-DC-SIGN-antibody and therefore express DC-SIGN. Moreover, the COS7-DC-SIGN cells are able to bind ICAM-3 whereas mock transfected COS7 cells are unable to bind ICAM-3 (Figure 4B). Binding of DC-SIGN expressed by COS7 could be completely inhibited by antibodies against ICAM-3 and DC-SIGN, and was  $\text{Ca}^{2+}$  dependent since EGTA blocks adhesion completely (Figure 4B).

It is concluded that the ICAM-3 binding receptor expressed by DC (DC-SIGN) is identical to the placenta HIV gp120 binding C-type lectin (Curtis et al., 1992), a type II transmembrane protein consisting of 404 aa with three distinct domains. The N-terminal cytoplasmic domain of 40 aa residues is separated by a hydrophobic stretch of 15 aa from a region which consists of seven complete and one incomplete tandem repeat of nearly identical sequence. The remaining C-terminal region (Cys253-Ala404) shows homology to  $\text{Ca}^{2+}$ -dependent (C-type) lectins (Figure 3B).

#### Example 5: DC-SIGN is specifically expressed by DC.

Flowcytometric analysis of an extensive panel of hematopoietic cells with the AZN-D1/D2 antibodies demonstrates that the antigen is preferentially expressed by DC (Table 1). All the hematopoietic cells tested were negative for DC-SIGN expression except for DC. Furthermore, a RT-PCR analysis confirms that the mRNA encoding DC-SIGN is specifically transcribed in DC which is in accordance with the expression pattern of the DC-SIGN protein (Table 1).

To further investigate the expression of DC-SIGN in-vivo, immunohistochemical analysis of secondary lymphoid tissues with the anti-DC-SIGN antibodies was performed. These tissues are known to contain dendritic cells. Sections of tonsils and lymph nodes contained DC-SIGN expressing cells, which were predominantly observed in the T cell area of both tonsils and lymph nodes (Figure 5). Figure 5 shows the tissue distribution of DC-SIGN: Immunohistochemical analysis of the expression of DC-SIGN in tonsils and lymph node sections (OMx100). Sections were fixed with acetone and the nuclear staining was performed with Hematein. Staining of DC-SIGN was performed with

AZN-D1. The germinal center (GC), T- (T) and B-cell (B) areas are depicted.

Consistent with the distribution and morphology of dendritic cells, DC-SIGN expressing cells are not detected in the germinal centres and the mantle zone of the lymphoid tissues (Figure 5). Staining of serial sections for CD3 and CD14 confirmed that the DC-SIGN expressing cells are distinct from T cells and monocytes (data not shown) as was also demonstrated by both flowcytometric analysis and RT-PCR of these cells (Table 1).

**Example 6: DC-SIGN /ICAM-3 interactions mediate transient DC-T lymphocyte clustering.**

To demonstrate that DC bind to ICAM-3 expressing transfectants in a DC-SIGN dependent manner, the capacity of the leukemic cell line K562 transfected with the cDNA encoding ICAM-3 (K562-ICAM-3) to bind to DC was investigated. As shown in Figure 6A, DC cluster with K562-ICAM-3 in a DC-SIGN dependent manner, since the interaction can be blocked by anti-DC-SIGN antibodies. No clustering was observed between DC and K562 demonstrating that ICAM-3 is the ligand for DC-SIGN. Figure 6 shows that DC-SIGN mediated adhesion of DC to ICAM-3 is involved in the DC-T-lymphocyte interaction:

- 6A: DC-SIGN mediated adhesion of DC to ICAM-3 is dependent on an intact cytoskeleton. Adhesion of DC to ICAM-3 beads was determined with (open box) or without (filled box) blocking DC-SIGN antibody AZN-D1 in the presence of Cytochalasin D, which was titrated in various concentrations. A representative experiment of 2 experiments is shown (SD<5%).

- 6B: Heterotypic cell clustering of DC with K562-ICAM-3 cells. K562 and K562 cells stable transfected with the cDNA encoding ICAM-3 (K562-ICAM-3) were labeled with the red dye HE (hydroethidine). DC were labeled with the green dye SFDA. K562 and K562-ICAM-3 were incubated with DC ( $50 \times 10^3$  cells/cell type) with or without blocking anti-DC-SIGN antibody (AZN-D1; 10 min. pre-incubation) at 37°C. At different time points the cells were fixed with paraformaldehyde (0.5%)

and the heterotypic cell clustering was measured flow-cytometrically. A representative experiment of 2 experiments is given.

- 6C: Dynamic cell clustering of DC with resting PbL is mediated by DC-SIGN. DC ( $50 \times 10^3$  cells) were pre-incubated with/without the anti-DC-SIGN antibodies AZN-D1 and AZN-D2 ( $10 \mu\text{g/ml}$ ) for 10 min. at RT. Allogeneic PBL ( $1 \times 10^6$  cells), labeled with the fluorescent dye Calcein-A ( $25 \mu\text{g}/10^7$  cells/ml for 30 min. at  $37^\circ\text{C}$ ), were added and the cell mixture was incubated at  $37^\circ\text{C}$ . The clustering was measured by flow-cytometry. A representative experiment out of 2 is shown.

- 6D: The DC-SIGN-ICAM-3 interaction is important in DC-induced T-cell proliferation. Allogeneic responder T-lymphocytes ( $100 \times 10^3$ ) were added to DC-stimulators ( $1.5 \times 10^3$ ) in the presence of blocking antibodies ( $20 \mu\text{g/ml}$ ) against LFA-3 (TS2/9) and DC-SIGN (AZN-D1, AZN-D2). The cells were cultured for 4 days. On day 4 the cells were pulsed for 16 h with [ $^3\text{H}$ ]methyl-thymidine and the uptake was determined. The results are expressed as the mean percent of CPM from triplicate wells.

DC-SIGN dependent clustering is transient, with a maximum at 60 minutes indicating that DC-SIGN-ICAM-3 interactions may be actively regulated by the DC. Furthermore, this phenomenon allows DC to transiently interact with multiple naive T cells until the interaction is strengthened after TCR engagement.

To test this it was investigated whether clustering of DC to T cells is mediated by DC-SIGN and whether this interaction is also transient. DC were incubated with resting allogeneic T cells (DC:T cell, 1:20) and the DC-T cell clustering was determined. As shown in Figure 6B, the clustering of DC with T cells is transient and reaches a maximum after 20 min (32%). Furthermore, the DC-T cell interaction can be inhibited (approximately 50%) by anti-DC-SIGN antibodies suggesting that the DC-T cell clustering is also mediated by other surface receptors. Thus, the DC-T cell clustering is indeed transient and partly mediated by DC-SIGN/ICAM-3 interactions. Similarly, Figure 8 shows that DC-SIGN binds not only with K562 cells expressing cDNA encoding ICAM-3, but also to K562 cells expressing cDNA encoding ICAM-3, and that said binding can

be inhibited by both mannan as well as anti DC-SIGN antibodies.

**Example 7: Proliferation of resting T cells induced by allogeneic DC is DC-SIGN dependent.**

5           As DC-SIGN binding to ICAM-3 is important for the clustering of DC with T cells, the role of DC-SIGN in DC induced T cell proliferation was also investigated. Resting T lymphocytes were stimulated with allogeneic DC in the presence or absence of the blocking anti-DC-SIGN antibodies. As shown in Figure 6C, the anti-DC-SIGN antibodies AZN-D1 and AZN-D2 both inhibited the T-lymphocyte proliferation for more than 75%. Similarly, antibodies against the co-stimulatory molecule LFA-3, which binds to CD2 on T cells and is also known to mediate T cell proliferation, inhibit T cell proliferation. A combination of anti-LFA-3 and anti-DC-SIGN antibodies completely inhibits T-cell proliferation (Figure 6C).

**Example 8: DC-SIGN is involved in the HIV-1 infection of DC.**

15           As it was demonstrated hereinabove that DC-SIGN is identical to the placenta HIV gp120 binding lectin and is abundantly expressed by DC, DC-SIGN might facilitate HIV-1 entry into DC. To investigate this, DC was pulsed with HIV-1 and productive infection in DC-T cell co-cultures was measured. DC harvested after 7 days of culture in the presence of IL-4 and GM-CSF expressed low levels of CD4 (Blauvelt et al., 1997; Granelli-Piperno et al., J Exp Med 20 184(6), 2433-8, 1996) and high levels of DC-SIGN (Figure 7). As shown in Figure 7 a strong productive infection takes place when DC are pulsed with HIV-1 for 2 hours, washed and cultured in the presence of activated PBMC cells. By day 3 of the DC-T cell co-culture the p24 Gag protein, a measure for HIV-1 replication, starts to accumulate in the medium (Figure 7) demonstrating that HIV-1 is efficiently replicated in the co-culture, similar as has been shown by others (Blauvelt et al., 1997; Granelli-Piperno et al., 1998; Granelli-Piperno et al. Curr Biol 9(1), 21-29, 1999). However, when DC prior to the HIV-1 pulse are pre-incubated with anti-DC-SIGN antibodies and incubated with activated PBMC,

HIV-1 replication is inhibited for more than 75%, as shown at day 3 and 5 of DC-T cell co-culture (Figure 7). When DC were incubated with anti-DC-SIGN antibodies after pulsing with HIV-1, efficient HIV-1 replication was still observed in the DC-T cell co-culture (Figure 7). These findings demonstrate that anti-DC-SIGN antibodies block HIV-1 infection through inhibition of HIV-1 binding to DC and not the HIV-1 transmission from DC to T cells, indicating that DC-SIGN act as a major receptor for HIV-1 on DC. Thus, DC-SIGN is highly expressed on DC and functions as a DC specific receptor for both ICAM-3 and HIV-1.

From the above experimental results, it can *inter alia* be concluded that the initial interaction of DC with T lymphocytes is antigen-independent and transient. This transient nature provides DC with the capacity to interact with a multitude of T cells until a productive TCR engagement is made. Until now, the mechanism by which this transient process is initiated has been unclear. Herein, it is demonstrated that the interaction of a novel DC specific receptor, DC-SIGN, with ICAM-3 mediates this transient DC-T cell interaction. DC-SIGN is abundantly expressed by DC and it was shown that DC-SIGN serves as a major HIV-1 receptor on DC.

An important role for DC during the course of HIV-1 infection is the ability to spread HIV-1 to T cells, promoting extensive replication that leads to the death of CD4+ T cells (Cameron et al., 1992; Cameron: AIDS Res Hum Retroviruses 10(1), 61-71, 1994). Productive HIV-1 infection of DC has been clearly demonstrated and depends on the development stage of the DC (Granelli-Piperno et al., 1998). Immature DC, cultured from monocytes in the presence of IL-4 and GM-CSF, are productively infected by M-tropic HIV-1 strains (Granelli-Piperno et al., 1996; Granelli-Piperno et al., 1998)(Blauvelt et al., 1997) whereas both M- and T-tropic HIV-1 entry into mature DC does not lead to a productive infection (Granelli 1998). However, HIV-1 entry into both types of DC does lead to an explosive replication upon co-culturing with either resting or activated T cells (Granelli 1998, 1999). The initial events in HIV-1 infection of target cells include receptor binding and membrane fusion. This process is initiated by the high affinity binding of the envelope glycoprotein gp120 to CD4. However, CD4

alone is not sufficient to initiate fusion, chemokine receptors such as CCR5 and CXCR4 are required as co-receptors for the final fusion event to occur (reviewed by Littman et al., 1998)(Dragic et al., Nature 381(6584), 667-73, 1996; Feng et al., Science 272(5263), 872-7, 1996). DC express low amounts of CD4 whereas high levels of DC-SIGN are expressed on the cell surface. It has been suggested that productive infection of DC and its ability to capture and subsequently transmit HIV-1 are mediated through separate pathways. Productive infection of DC is mediated by a CD4-dependent pathway whereas HIV-1 can be captured by DC through a CD4-independent pathway which still enables DC to transmit HIV-1 to T cells (Blauvelt et al. (1997)). Herein, it was shown that DC-SIGN specifically mediates entry of HIV-1 into DC, as was measured by lack of productive infection in the DC-T cell co-culture upon preincubation of DC with anti-DC-SIGN antibodies prior to the HIV-1 pulse. Anti-DC-SIGN antibodies do not completely inhibit HIV-1 entry into DC. This DC-SIGN-independent pathway is probably mediated by CD4 ( ) which is expressed at low levels on DC. These results confirm the presence of both a CD4-dependent and independent pathway for viral entry into DC. Various adhesion molecules have been shown to be able to inhibit transmission of HIV-1 from DC to T cells through interference of DC-T cell contact (Tsunetsugu-Yokota et al., 1997). Anti-DC-SIGN antibodies could not prevent HIV-1 transmission to T cells when anti-DC-SIGN antibodies were added after the HIV-1 pulse to inhibit the DC-T cell interaction. These data indicate that DC-SIGN serves as a major receptor for HIV-1 entry into DC. The fact that DC express high levels of DC-SIGN and low levels of CD4 (Figure 7) further demonstrates that HIV-1 entry into DC is predominantly mediated by DC-SIGN. The discovery of DC-SIGN as a HIV-1 receptor could be important in a better understanding of HIV-1 entry into DC. Furthermore, the inhibition of HIV-1 infection observed in the presence of anti-DC-SIGN antibodies will enable the development of anti-DC-SIGN antibodies in therapeutic strategies against viral infection and regional spread of HIV-1.

DC constitute an heterogeneous population of cells which are present at trace levels in various tissues. To better define the different populations a lot of

effort has gone into the generation of antibodies that are directed against DC lineage specific cell surface molecules. So far only a few antibodies have been generated which recognize human DC specific antigens ((Hock et al., Immunol. 83, 573-581, 1994), (de Saint-Vis et al., Immunity 9(3), 325-36, 1998)(Hart et al., 1997). DC-SIGN can now been added to this list of human DC specific antigens since it was demonstrated herein that at the protein as well as mRNA level, of all hematopoietic cells tested, only DC express DC-SIGN (Table 1). In situ DC-SIGN is exclusively expressed by DC subsets present in the T cell area of tonsils and lymph nodes. These mature DC are very potent in the activation of naive T cells. Therefore, DC-SIGN expression in situ correlates with its function as an important mediator of DC-T cell clustering and subsequent T cell activation.

Activation of resting T lymphocytes by antigen presenting cells is a critically important step in the acquired immune response. Located in most tissues, DC capture and process antigens, and migrate to lymphoid tissues where they interact with and activate naive antigen-specific T cells. T cells are directed by chemokines to these sites of antigen presentation. Recently, a DC specific chemokine DC-CK1 was identified which specifically attracts naive T cells to immune initiation sites (Adema et al., Nature 387, 713-717, 1997). Upon arrival in secondary lymphoid tissues, T cells interact with DC and activation occurs after TCR recognition of peptides bound to MHC molecules. However, since the affinity of the TCR for the antigen presented by MHC molecules is very low and the number of specific MHC-peptide complexes on APC is limited, the interaction of TCR with antigen is usually insufficient to drive the formation of intimate membrane contact between DC and T-lymphocyte necessary for full activation.

To date LFA-1 was the most important receptor for ICAM-3 on DC. However, its role in ICAM-3 binding has now become disputable due to the discovery herein of DC-SIGN. It was demonstrated that adhesion of DC to ICAM-3 is completely mediated by DC-SIGN. DC-SIGN is more abundantly expressed by DC than LFA-1 (Figure 2B). Furthermore, LFA-1 is inactive on DC (Figure 2C) and its affinity for ICAM-3 is much lower than that of DC-SIGN for ICAM-3. These data clearly demonstrate that DC-SIGN is the primary receptor for

ICAM-3 on DC. The function for DC-SIGN on DC was further clarified by the finding that anti-DC-SIGN antibodies *partially* inhibited transient DC-T cell clustering. Therefore, DC-SIGN is involved in the initial DC-T cell interaction in the immune response. A role which was previously attributed to LFA-1. The transient nature of the DC-T cell interaction mediated by DC-SIGN enables DC to interact with a large number of resting T cells, until a productive TCR mediated interaction is made upon which the interaction is stabilized. The importance of the DC-SIGN-ICAM-3 interaction is further underscored by the finding that antibodies against DC-SIGN are able to inhibit allogeneic DC induced T-lymphocyte proliferation. Moreover, the combination of antibodies against DC-SIGN and LFA-3, a known co-stimulatory molecule (), almost completely inhibit T-lymphocyte proliferation. Therefore, transient high affinity adhesion of DC-SIGN to ICAM-3 plays an important role in the initial antigen-independent interaction between DC and naive T cells. Presumably, this initial high affinity interaction enables engagement of the TCR by the antigen bound to the MHC, which subsequently initiates several other adhesive interactions between DC and T cells, such as the LFA-1-ICAM-1 interaction. Since LFA-1 is inactive on T cells, activation of the TCR/CD3 complex after antigen presentation by DC will result in activation of LFA-1 and subsequent strong binding of LFA-1 to ICAM-1 expressed on DC (). Strengthening of the interaction between DC and T cell via multiple contacts will then lead to full activation of the T lymphocyte by the DC stimulator ().

In conclusion, a novel ICAM-3 receptor on DC was identified, designated DC-SIGN, which receptor is specifically expressed by human DC and is involved in the initial transient DC-T cell interaction necessary for initiating an immune response. Interestingly, DC-SIGN is also able to bind the HIV envelope protein gp120 and to facilitate HIV-1 entry into DC. Various therapeutic and prophylactic possibilities and techniques, based upon the findings disclosed herein, will suggest themselves to the skilled person.

**Example 9: Experimental procedures.**

**Ex. 9A: Antibodies**

The following antibodies were used: KIM185 (anti- $\beta$ 2 integrin, (Andrew et al., Eur.J.Immunol. 23, 2217-2222, 1993), AZN-L19 (anti- $\beta$ 2 integrin, ), NKI-L15 (anti- $\alpha$ L, (Keizer et al., Eur.J.Immunol. 15, 1142-1147, 1985)), AIIB2 (anti- $\beta$ 1 integrin, (Da Silva et al., J.Immunol. 143, 617-622, 1989)), CBR-IC3/1 and CBR-IC3/2 (anti-ICAM-3 (de Fougerolles et al., J.Exp.Med. 177, 1187-1192, 1993)), CD14 (WT14 ()), CD4 (wt4 ()). The anti-DC-SIGN antibodies AZN-D1 and AZN-D2 were obtained by immunizing BALB/c mice with DC and subsequently screening the hybridoma supernatants for the ability to block adhesion of DC to ICAM-3 as measured by the fluorescent beads adhesion assay.

**Ex. 9B: Cells**

DC were cultured from monocytes as described (Sallusto and Lanzavecchia, J.Exp.Med. 179, 1109-1118, 1994; Romani et al., J.Exp.Med. 180, 83-93, 1994). Briefly, monocytes were isolated from fresh PBMC by an adherence step. The monocytes were cultured in the presence of IL-4 (Schering-Plough, Brussels, Belgium; 500 U/ml) and GM-CSF (Schering-Plough, Brussels, Belgium; 1000 U/ml) for 7 days. At day 4 fresh cytokines were added. At day 7 the phenotype of the cultured DC was confirmed by flowcytometric analysis of the expression of MHC class I and II, CD1a, p150,95 and CD80. Stable K562 transfectants expressing ICAM-3 (K562-ICAM-3) were generated by transfection of K562 with 10  $\mu$ g PCRII ICAM-3 R1 plasmid (gift from Dr D. Simmons) and 2  $\mu$ g PGK-hyg vector (te Riele et al 1990) by electroporation as described (Lub et al., Mol.Biol Cell 8, 719-728, 1997). Resting T cells (>90% CD3 positive) were obtained by centrifugal elutriation of PBMC from bone marrow of healthy donors, as described (Figdor et al., J.Immunol.Methods 68, 73-87, 1984).

**Ex. 9C: Radiolabeling, immunoprecipitation and protein sequence analysis.**

Cells were surface labeled with Na<sup>125</sup>I (Amersham, Buckinghamshire, UK) through the lactoperoxidase method (Pink and Ziegler, 1979, in: *Research Methods in Immunology*, L. Lefkovits and B. Pernis, eds. (New York: Academic Pres), pp.

169-180.). DC were lysed for 1 hr at 4°C in lysis buffer (1% NP-40, 50 mM tri-ethanolamine (pH 7.8), 150 mM NaCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 1 mM PMSF, 0.02 mg/ml leupeptin). Nuclear debris was removed from the lysate by centrifugation at 13,000 g for 15 min at 4°C. Pre-cleared lysates were incubated for 3 hr with a specific mAb covalently coupled to Protein A-sepharose CL-4B beads (Pharmacia Fine Chemicals, Piscataway, NJ). The immunoprecipitates were extensively washed with lysis buffer and analysed by SDS-PAGE according to a modification of the Laemmli procedure (Laemmli, Nature 227, 680-685, 1970). Tryptic digestion of the excised protein, purification of the resulting peptides and sequence analysis was performed by Eurosequence BV (Groningen, The Netherlands).

#### Ex. 9D: Isolation and expression of the cDNA encoding DC-SIGN.

Total RNA was isolated by an acidic guanidinium isothiocyanate-phenol-chloroform procedure (Chomczynski and Sacchi, Anal Biochem 162(1), 156-9, 1987 ). The cDNA encoding the placenta gp120 binding C-type lectin was amplified by RT-PCR on total RNA from DC. PCR primers were based on the nucleotide sequence of the placenta gp120 binding C-type lectin (accession no. M98457, (Curtis et al., 1992)) and the nucleotide sequences (5' to 3') are as follows: XF29, AGAGTGGGGTGACATGAGTG; XR1265, GAAGTTCTGCTACGCAGGAG. The PCR fragment was cloned into the pGEM-T vector (Promega, Madison WI) and sequenced. The nucleotide sequence of the cloned cDNA was identical to that of placenta gp120 binding C-type lectin (Curtis et al., 1992). The cDNA was subsequently cloned into the eukaryotic expression vector pRc/CMV (pRc/CMV-DC-SIGN) and COS7 cells were transiently transfected with pRc/CMV-DC-SIGN using the DEAE dextran method (Seed and Aruffo, Proc.Natl.Acad.Sci.U.S.A. 84, 3365-3369, 1987).

#### Ex. 9E: Fluorescent beads adhesion assay

Carboxylate-modified TransFluorSpheres (488/645 nm, 1.0 µm; Molecular Probes, Eugene, OR) were coated with ICAM-1 Fc and ICAM-3 Fc as described

previously (Geijtenbeek et al., 1999 submitted). Briefly, 20  $\mu$ l streptavidin (5 mg/ml in 50 mM MES-buffer) was added to 50  $\mu$ l TransFluorSpheres. 30  $\mu$ l EDAC (1.33 mg/ml) was added and the mixture was incubated at RT for 2h. The reaction was stopped by the addition of glycine to a final concentration of 100 mM.

5 The streptavidin-coated beads were washed three times with PBS (50 mM phosphate, 0.9% NaCl pH 7.4) and resuspended in 150  $\mu$ l PBS, 0.5% BSA (w/v). The streptavidin-coated beads (15  $\mu$ l) were incubated with biotinylated goat-anti-human anti-Fc Fab2 fragments (6  $\mu$ g/ml) in 0.5 ml PBA for 2 hours at 37°C. The beads were washed once with PBS, 0.5% BSA and incubated with human IgG1 Fc

10 fused ligands (ICAM-1 Fc, VCAM-1 Fc; 250 ng/ml) in 0.5 ml overnight at 4°C. The ligand-coated beads were washed, resuspended in 100  $\mu$ l PBS, 0.5% BSA and stored at 4°C. ICAM-1 Fc and ICAM-3 Fc consist of the extracellular part of the protein fused to a human IgG1 Fc fragment (provided by Dr D. Simmons). The fluorescent beads adhesion assay was performed as described by Geijtenbeek et al.

15 (submitted). Briefly, cells were resuspended in Tris-Sodium-BSA buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1mM  $\text{CaCl}_2$ , 2 mM  $\text{MgCl}_2$ , 0.5% BSA;  $5 \times 10^6$  cells/ml). 50.000 cells were pre-incubated with/without blocking mAb (20  $\mu$ g/ml) for 10 min at RT in a 96-wells V-shaped bottom plate. Ligand-coated fluorescent beads (20 beads/cell) and different stimuli/inhibitors were added and the

20 suspension was incubated for 30 min at 37°C. The cells were washed and resuspended in 100  $\mu$ l TSA. Adhesion was determined by measuring the percentage of cells, which have bound fluorescent beads, by flowcytometry using the FACScan (Becton and Dickinson & Co., Oxnard, CA).

#### 25 Ex. 9F: Heterotypic cell clustering assays

Clustering between DC and ICAM-3 expressing cells was assessed by flowcytometry. DC and ICAM-3 expressing cells ( $2 \times 10^6$  cells/ml) were labeled respectively with sulfofluorescein (Molecular Probes, Eugene, OR; 50  $\mu$ g/ml) and hydroethidine (Molecular Probes, Eugene, OR; 40  $\mu$ g/ml) for 1 hour at 37°C.

30 After washing, DC and the ICAM-3 expressing cells were mixed ( $50 \times 10^3$  cells each) and incubated at 37°C. At different time points the cells were fixed with

paraformaldehyde (0.5%) and the heterotypic cell clustering was measured by flowcytometry using the FACScan (Becton and Dickinson & Co., Oxnard, CA).

Clustering between DC with resting T cells was assessed by a different method. DC ( $50 \times 10^3$  cells) were pre-incubated with/without the anti-DC-SIGN antibodies AZN-D1 and AZN-D2 (10  $\mu$ g/ml) for 10 min. at RT. Allogeneic PBL ( $1 \times 10^6$  cells), labeled with the fluorescent dye Calcein-A (Molecular Probes, Eugene, OR;  $25 \mu$ g/ $10^7$  cells/ml for 30 min. at  $37^\circ$ ), were added and the cell mixture was incubated at  $37^\circ\text{C}$ . The clustering was determined by measuring percentage of DC which have bound fluorescent T cells by flowcytometry using the FACScan (Becton and Dickinson & Co., Oxnard, CA).

#### Ex. 9G: DC-induced T cell proliferation assay

Allogeneic responder T-lymphocytes ( $100 \times 10^3$ ) were added to DC-stimulators ( $1.5 \times 10^3$ ) in the presence of blocking antibodies (20  $\mu$ g/ml). The cells were cultured for 4 days. On day 4 the cells were pulsed for 16 h with [ $^3\text{H}$ ]methyl-thymidine (1.52 TBq/mmol, 0.5  $\mu$ Ci/well; Amersham, Buckinghamshire, UK) and the uptake was quantified.

#### Ex. 9H: HIV-1 infection of DC

HIV-1<sub>Ba-L</sub> was grown to high titer in monocyte-derived macrophages (MDM). Seven days after titration of the virus stock on MDM,  $\text{TCID}_{50}$  was determined with a p24 antigen ELISA ((Diagnostics Pasteur, Marnes la Coquette, France) and estimated as  $10^4$ /ml. DC ( $50 \times 10^3$ ), pre-incubated with antibodies (50  $\mu$ g/ml) for 20 min. at RT, were pulsed for 2 h. with wild-type HIV-1<sub>Ba-L</sub> (at a multiplicity of infection of  $10^3$  infectious units per  $10^5$  cells), washed and co-cultured with PHA/IL-2 activated PBMC ( $50 \times 10^3$ ). Supernatants were collected 3 and 5 days after DC-T cell co-culture and p24 antigen levels were measured by a p24 antigen ELISA (Diagnostics Pasteur, Marnes la Coquette, France). PBMC were activated by culturing them in the presence of IL-2 (10 U/ml) and PHA (10  $\mu$ g/ml).

**Ex. 9I: Immunohistochemical analysis**

Cryosections (8  $\mu$ m) of tonsils and lymph nodes were fixated in 100% acetone (10 min), washed with PBS and incubated with the first antibody (10  $\mu$ g/ml) for 60 min at RT. After washing, the final staining was performed with the ABC-AP Vectastain kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. Nuclear staining was performed with hematein ().

Table 1: Expression level of DC-SIGN on hematopoietic cells as determined by flowcytometric analyses and RT-PCR.

Cell-type	DC-SIGN expression*	DC-SIGN mRNA†
monocytes	-	-
DC day 7	+++	+
PBL	-	-
T cells	-	-
B cells	-	-
B-cells (tonsils)\$	-	n.d.
Thymocytes	-	-
Granulocytes	-	-
CD34+ cells	-	n.d.
PBMC (activated#)	-	-
T cell lines†	-	-
monocytic cell lines††	-	-

\* mean fluorescence: -=<20, +++ >400 (staining with AZN-D1)

† RT-PCR with the DC-SIGN specific primers XF29 and XR1265 on total RNA isolated from the different cells

\$ isolated from tonsils

# activated with PHA (10  $\mu$ g/ml) and IL-2 (10 U/ml) for 2 days

† T cell lines : HSB, PEER, CEM and Jurkat

†† monocytic cell lines: THP-1, MM6 and U937

n.d., not determined

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## 5 (i) APPLICANT:

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15

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

20

(D) SOFTWARE: PatentIn Release #1.0. Version #1.30

(ii) TITLE OF INVENTION: COMPOSITION AND METHOD FOR MODULATING  
DENDRITIC CELL-T CELL INTERACTION

25

(iii) NUMBER OF SEQUENCES: 2

## (2) INFORMATION FOR SEQ ID NO:1:

30

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1215 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

40

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

45 ATGAGTGACT CCAAGGAACC AAGACTGCAG CAGCTGGGCC TCCTGGAGGA GGAACAGCTG 60  
AGAGGCCTTG GATTCCGACA GACTCGAGGA TACAAGAGCT TAGCAGGGTG TCTTGGCCAT 120  
GGTCCCCTGG TGCTGCAACT CCTCTCCTTC ACGCTCTTGG CTGGGCTCCT TGTCCAAGTG 180  
50 TCCAAGGTCC CCAGCTCCAT AAGTCAGGAA CAATCCAGGC AAGACGCGAT CTACCAGAAC 240  
CTGACCCAGC TTAAAGCTGC AGTGGGTGAG CTCTCAGAGA AATCCAAGCT GCAGGAGATC 300  
55 TACCAGGAGC TGACCCAGCT GAAGGCTGCA GTGGGTGAGC TTCCAGAGAA ATCTAAGCTG 360

CAGGAGATCT ACCAGGAGCT GACCCGGCTG AAGGCTGCAG TGGGTGAGCT TCCAGAGAAA 420  
 TCTAAGCTGC AGGAGATCTA CCAGGAGCTG ACCTGGCTGA AGGCTGCAGT GGGTGAGCTT 480  
 5 CCAGAGAAAT CTAAGATGCA GGAGATCTAC CAGGAGCTGA CTCGGCTGAA GGCTGCAGTG 540  
 GGTGAGCTTC CAGAGAAATC TAAGCAGCAG GAGATCTACC AGGAGCTGAC CCGGCTGAAG 600  
 10 GCTGCAGTGG GTGAGCTTCC AGAGAAATCT AAGCAGCAGG AGATCTACCA GGAGCTGACC 660  
 CGGCTGAAGG CTGCAGTGGG TGAGCTTCCA GAGAAATCTA AGCAGCAGGA GATCTACCAG 720  
 GAGCTGACCC AGCTGAAGGC TGCAGTGGAA CGCCTGTGCC ACCCCTGTCC CTGGGAATGG 780  
 15 ACATTCTTCC AAGGAAACTG TTACTTCATG TCTAACTCCC AGCGGAACTG GCACGACTCC 840  
 ATCACCGCCT GCAAAGAAGT GGGGGCCCAG CTCGTCGTAA TCAAAAGTGC TGAGGAGCAG 900  
 20 AACTTCCTAC AGCTGCAGTC TTCCAGAAGT AACCGCTTCA CCTGGATGGG ACTTTCAGAT 960  
 CTAAATCAGG AAGGCACGTG GCAATGGGTG GACGGCTCAC CTCTGTTGCC CAGCTTCAAG 1020  
 CAGTATTGGA ACAGAGGAGA GCCCAACAAC GTTGGGGAGG AAGACTGCGC GGAATTTAGT 1080  
 25 GGCAATGGCT GGAACGACGA CAAATGTAAT CTTGCCAAAT TCTGGATCTG CAAAAAGTCC 1140  
 GCAGCCTCCT GCTCCAGGGA TGAAGAACAG TTTCTTTCTC CAGCCCCTGC CACCCCAAAC 1200  
 30 CCCCCTCCTG CGTAG 1215

2) INFORMATION FOR SEQ ID NO:2:

35

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 415 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

45 Met Ser Asp Ser Lys Glu Pro Arg Leu Gln Gln Leu Gly Leu Leu  
     1                    5                    10                    15  
 Glu Glu Glu Gln Leu Arg Gly Leu Gly Phe Arg Gln Thr Arg Gly  
                     20                    25                    30  
 50 Tyr Lys Ser Leu Ala Gly Cys Leu Gly His Gly Pro Leu Val Leu  
                     35                    40                    45  
 55 Gln Leu Leu Ser Phe Thr Leu Leu Ala Gly Leu Leu Val Gln Val  
                     50                    55                    60

Ser Lys Val Pro Ser Ser Ile Ser Gln Glu Gln Ser Arg Gln Asp  
 65 70 75  
 5 Ala Ile Tyr Gln Asn Leu Thr Gln Leu Lys Ala Ala Val Gly Glu  
 80 85 90  
 Leu Ser Glu Lys Ser Lys Leu Gln Glu Ile Tyr Gln Glu Leu Thr  
 95 100 105  
 10 Gln Leu Lys Ala Ala Val Gly Glu Leu Pro Glu Lys Ser Lys Leu  
 110 115 120  
 Gln Glu Ile Tyr Gln Glu Leu Thr Arg Leu Lys Ala Ala Val Gly  
 125 130 135  
 15 Glu Leu Pro Glu Lys Ser Lys Leu Gln Glu Ile Tyr Gln Glu Leu  
 140 145 150  
 Thr Trp Leu Lys Ala Ala Val Gly Glu Leu Pro Glu Lys Ser Lys  
 155 160 165  
 20 Met Gln Glu Ile Tyr Gln Glu Leu Thr Arg Leu Lys Ala Ala Val  
 170 175 180  
 25 Gly Glu Leu Pro Glu Lys Ser Lys Gln Gln Glu Ile Tyr Gln Glu  
 185 190 195  
 Leu Thr Arg Leu Lys Ala Ala Val Gly Glu Leu Pro Glu Lys Ser  
 200 205 210  
 30 Lys Gln Gln Glu Ile Tyr Gln Glu Leu Thr Arg Leu Lys Ala Ala  
 215 220 225  
 Val Gly Glu Leu Pro Glu Lys Ser Lys Gln Gln Glu Ile Tyr Gln  
 230 235 240  
 35 Glu Leu Thr Gln Leu Lys Ala Ala Val Glu Arg Leu Cys His Pro  
 245 250 255  
 40 Cys Pro Trp Glu Trp Thr Phe Phe Gln Gly Asn Cys Tyr Phe Met  
 260 265 270  
 Ser Asn Ser Gln Arg Asn Trp His Asp Ser Ile Thr Ala Cys Lys  
 275 280 285  
 45 Glu Val Gly Ala Gln Leu Val Val Ile Lys Ser Ala Glu Glu Gln  
 290 295 300  
 50 Asn Phe Leu Gln Leu Gln Ser Ser Arg Ser Asn Arg Phe Thr Trp  
 305 310 315  
 Met Gly Leu Ser Asp Leu Asn Gln Glu Gly Thr Trp Gln Trp Val  
 320 325 330  
 55

Asp Gly Ser Pro Leu Leu Pro Ser Phe Lys Gln Tyr Trp Asn Arg  
335 340 345

5 Gly Glu Pro Asn Asn Val Gly Glu Glu Asp Cys Ala Glu Phe Ser  
350 355 360

Gly Asn Gly Trp Asn Asp Asp Lys Cys Asn Leu Ala Lys Phe Trp  
365 370 375

10 Ile Cys Lys Lys Ser Ala Ala Ser Cys Ser Arg Asp Glu Glu Gln  
380 385 390

Phe Leu Ser Pro Ala Pro Ala Thr Pro Asn Pro Pro Pro Ala \*  
395 400 404

15

## C L A I M S

1. Use of a compound that binds to a C-type lectin on the surface of a dendritic cell, in the preparation of a composition for modulating, in particular  
5 reducing, the immune response in a animal, in particular a human or another mammal, with the proviso that the C-type lectin is not the DEC-205 receptor.

2. Use according to claim 1, in the preparation of a composition for modulating, in particular reducing, one or more interactions between a dendritic  
10 cell and a T cell.

3. Use according to claim 1 and/or 2, in the preparation of a composition for modulating, in particular reducing, the adhesion between a dendritic cell and a T cell, in particular between a C-type lectin on the surface of a dendritic cell and  
15 an ICAM receptor on the surface of a T cell, in particular an ICAM-2 or ICAM-3 receptor on the surface of a T cell.

4. Use according to any of claims 1-3, in the preparation of a composition for preventing or inhibiting immune responses to specific antigens, for inducing  
20 tolerance, for immunotherapy, for immunosuppression, for the treatment of autoimmune diseases, and/or for the treatment of allergy.

5. Use of a compound that binds or can bind to a C-type lectin on the surface of a dendritic cell, in the preparation of a composition for inhibiting the  
25 HIV infection of dendritic cells, in particular for inhibiting the adhesion of HIV surface protein (i.e gp 120) to the surface of a dendritic cell and thereby the entry of HIV into said dendritic cell.

6. Use of a compound that binds or can bind to a C-type lectin on the surface of a dendritic cell, in the preparation of a composition for inhibiting the  
30 transfer of HIV from infected dendritic cells to non-infected T cells.

7. Use of a combination of: 1) a compound that binds to a C-type lectin on the surface of a dendritic cell; and attached thereto: 2) an antigen or a fragment or part thereof; in the preparation of a composition for modulating, in particular generating, increasing and/or promoting, an immune response in a animal, in particular a human or another mammal, against said antigen.

8. Use according to claim 7, in which the antigen is covalently bonded to or fused with the compound that can bind to the C-type lectin.

9. Use according to claim 7 or 8, in which the antigen is chosen from cancer antigens which can be used to generate an immune response against tumor cells that contain or express said antigen, or antigens (as used in vaccines against infectious diseases,

10. Use according to any of claims 1-9, in which the compound that can bind to a C-type lectin on the surface of a dendritic cell is chosen from the group consisting of mannose carbohydrates such as mannan and D-mannose; fucose carbohydrates such as L-fucose; plant lectins such as concanavalin A; antibiotics such as pradimicin A; sugars such as N-acetyl-D-glucosamine and galactose; proteins such as gp120 and analogs or fragments thereof; and antibodies directed against a C-type lectin as expressed on the surface of a dendritic cell or a part, fragment or epitope thereof.

11. Use according to any of claims 1-9, in which the C-type lectin on the surface of a dendritic cell is a protein with the amino acid sequence of SEQ ID no.1, or a natural variant or equivalent thereof.

12. Use according to claim 10 or 11, in which the compound that can bind to a C-type lectin on the surface of a dendritic cell is a monoclonal antibody, preferably a monoclonal antibody directed against a C-type lectin with the amino acid sequence of SEQ ID no.1 or a natural variant or equivalent thereof; and/or a

part, fragment or epitope thereof.

13. Antibody, preferably monoclonal antibody, directed against a C-type lectin with the amino acid sequence of SEQ ID no.1 or Figure 9 or a natural variant or equivalent thereof; and/or a part, fragment or epitope thereof.

14. Antibody according to claim 13, being either AZN-D1 or AZN-D2.

15. Pharmaceutical composition, containing at least one antibody according to claim 13 or 14, and at least one further carrier, excipients, adjuvans and/or formulant known per se.

16. Combination of: 1) a compound that binds to a C-type lectin on the surface of a dendritic cell; and attached thereto; 2) an antigen or a fragment or part thereof.

17. Combination according to claim 16, in which the antigen is covalently bonded to or fused with the compound that can bind to the C-type lectin.

18. Combination according to claim 16 or 17, in which the antigen is chosen from cancer antigens which can be used to generate an immune response against tumor cells that contain or express said antigen, or antigens (as used in vaccines against) infectious diseases.

19. Combination according to any of claims 16-18, in which the compound that can bind to a C-type lectin on the surface of a dendritic cell is chosen from the group consisting of mannose carbohydrates such as mannan and D-mannose; fucose carbohydrates such as L-fucose; plant lectins such as concanavalin A; antibiotics such as pradimicin A; sugars such as N-acetyl-D-glucosamine and galactose; proteins such as gp120 and analogs or fragments

thereof; and antibodies directed against a C-type lectin as expressed on the surface of a dendritic cell or a part, fragment or epitope thereof.

5 20. Use of an antibody according to claim 13 or 14 in the detection of dendritic cells in a biological sample.

10 21. Use of an antibody according to claim 13 or 14 in the isolation, preparation and/or purification of dendritic cells from a biological sample or a culture medium.

15 22. Use of an antibody according to claim 13 or 14 in an assay for determining the presence and/or the expression of C-type lectins, in particular a C-type lectin with the amino acid sequence of SEQ ID no.1 or a natural variant or equivalent thereof; and/or a part, fragment or epitope thereof, in a biological sample.

23. Method for producing, isolating and/or purifying dendritic cells from a biological sample or a culture medium, comprising the steps of:

20 a) contacting a biological sample or a culture medium that contains dendritic cells with an antibody according to claim 13 or 14;  
b) separating the cells that bind to said antibody from cells that do not bind to said antibody, and optionally from any further constituents of the sample or medium;

and optionally further comprises the step of:

25 c) separating the cells that bind to the antibody from said antibody.

24. Method according to claim 23, in which the antibody is attached to a column or matrix, to (para)magnetic beads or to a similar solid support.

30

25. Method according to claim 23 or 24, in which the biological sample is a biological fluid, such as blood, plasma or lymph fluid.

26. Dendritic cells, obtained via the method of claim 23 or 24.

1/14

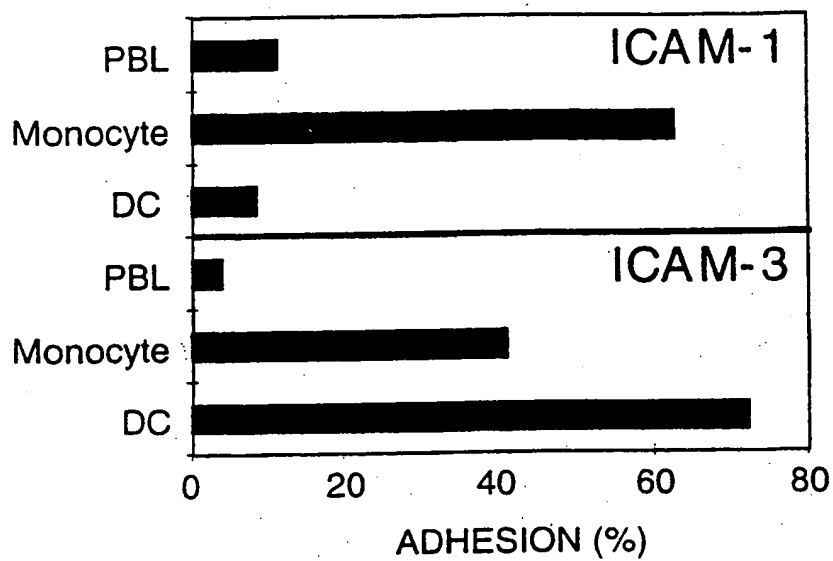
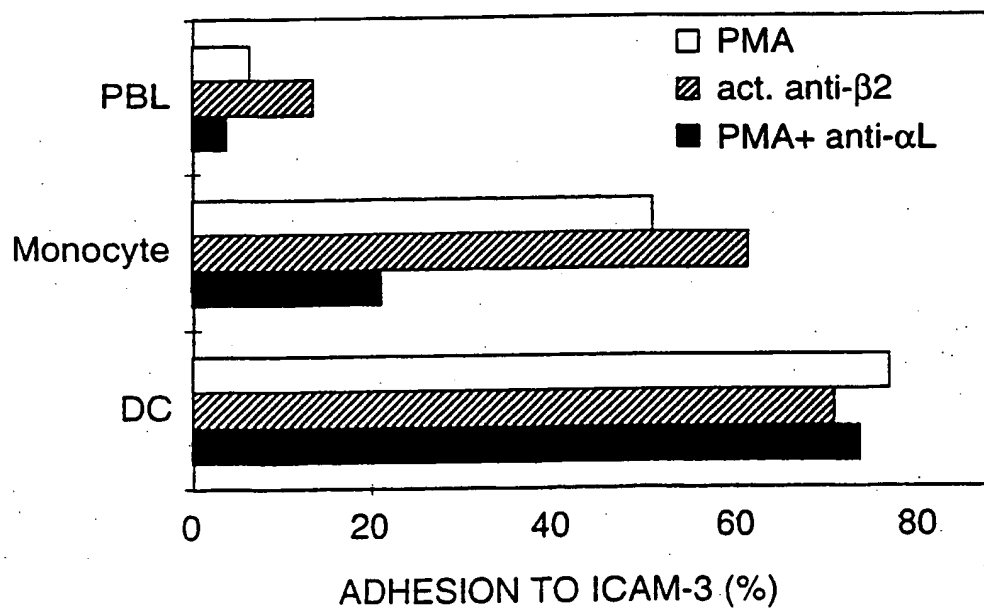
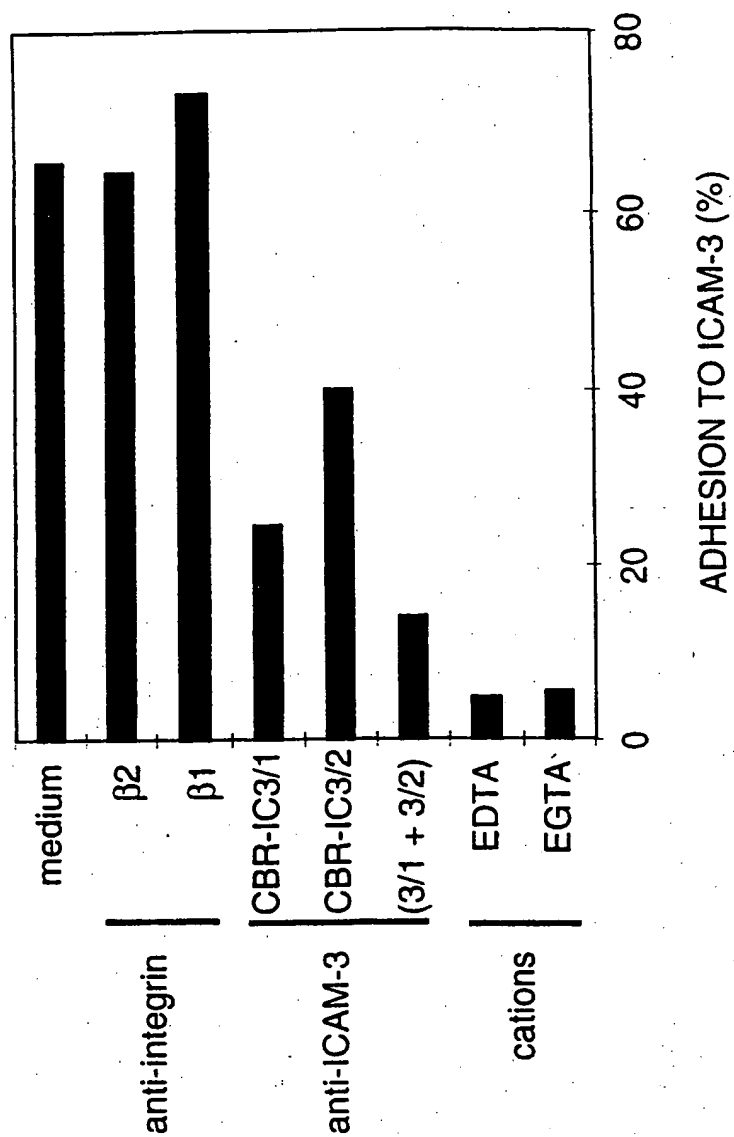
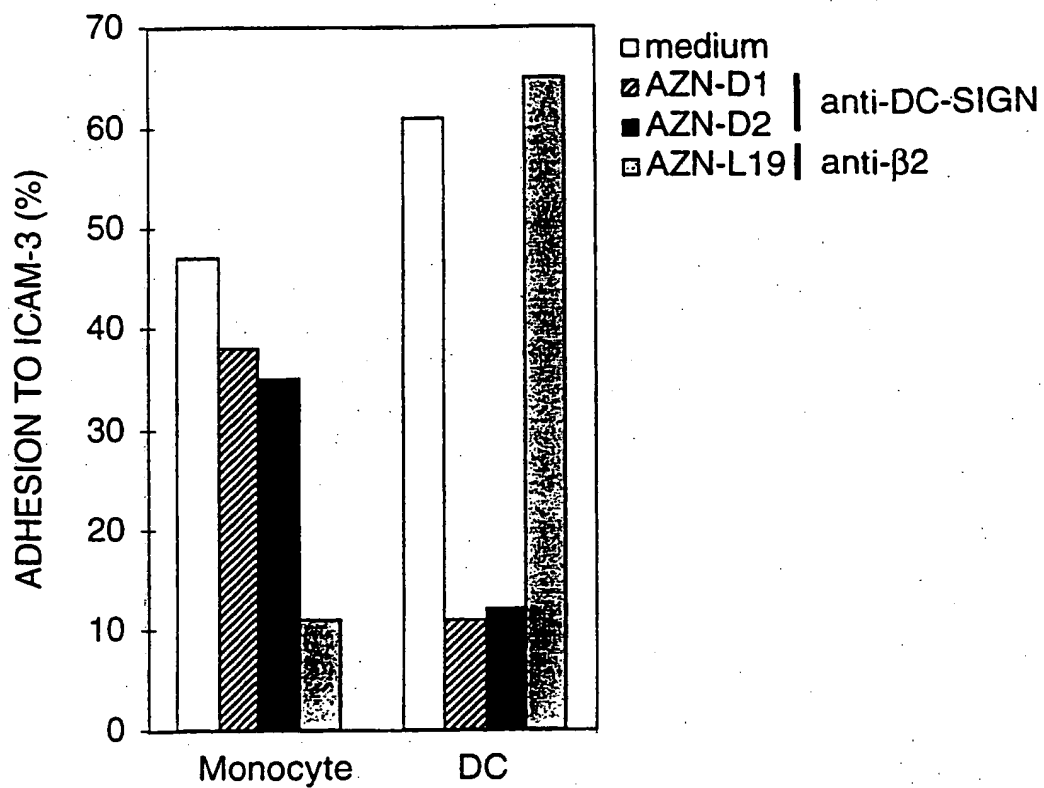
*Fig 1a**Fig 1b*

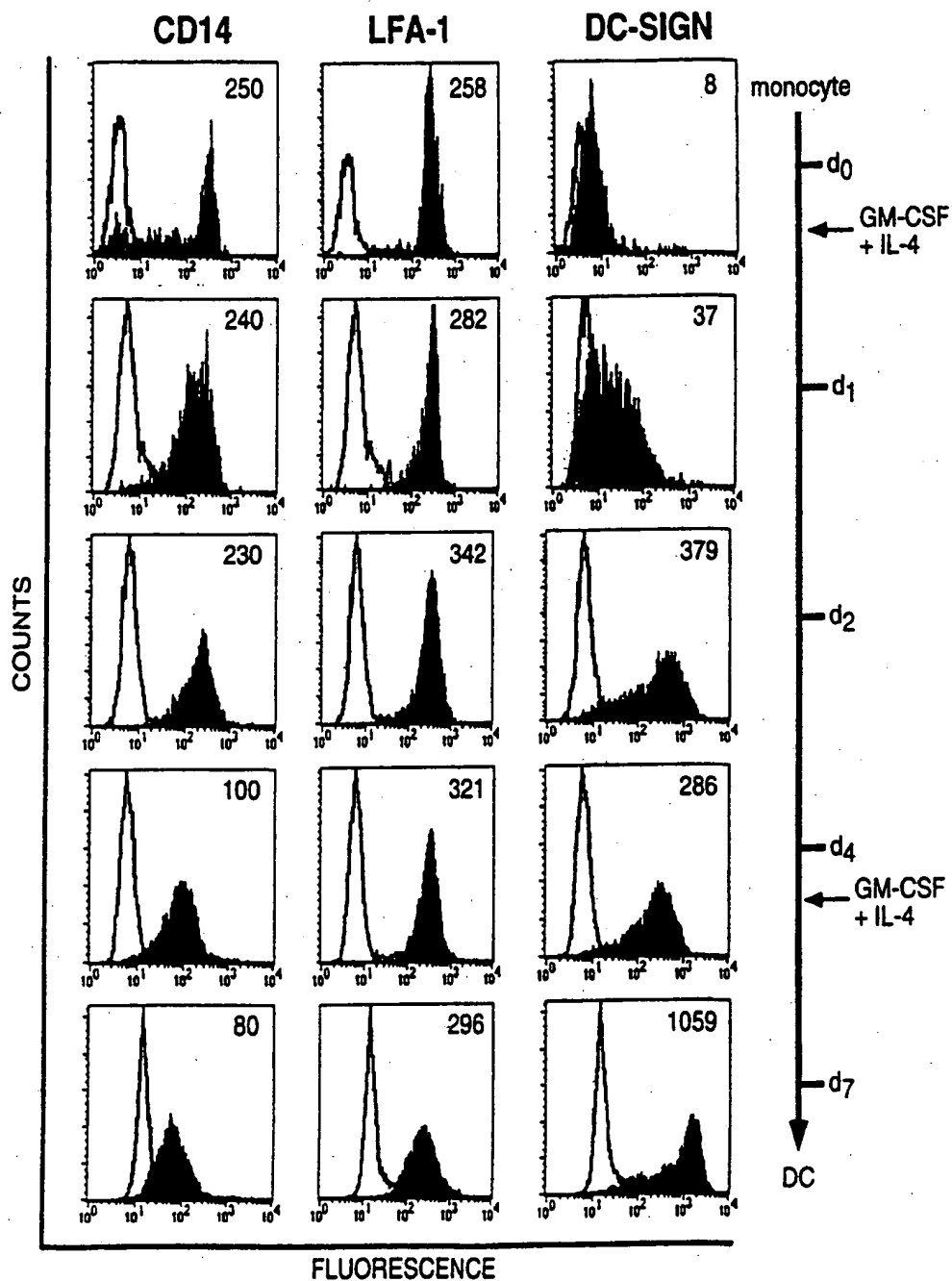
Fig 1c



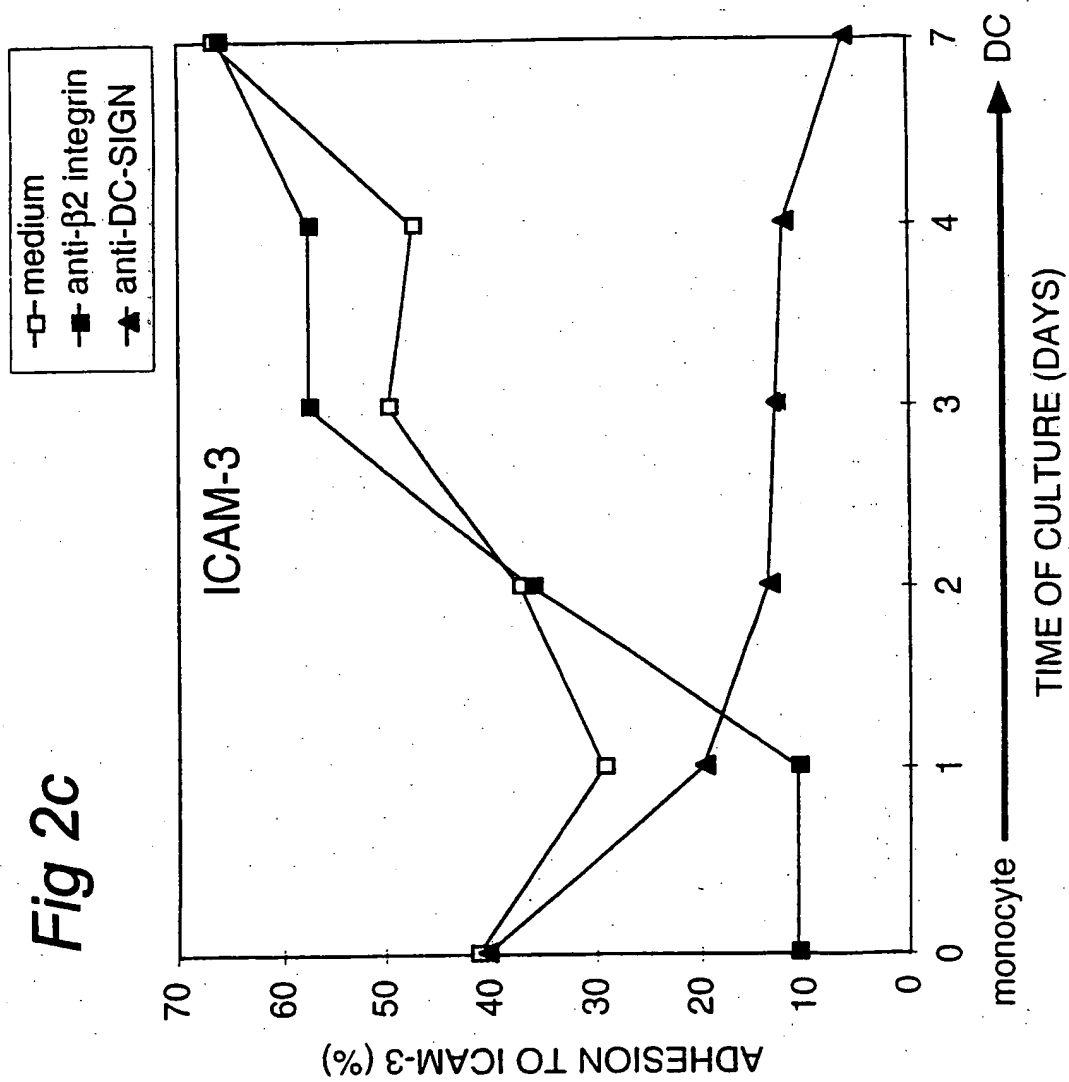
3/14

*Fig 2a*

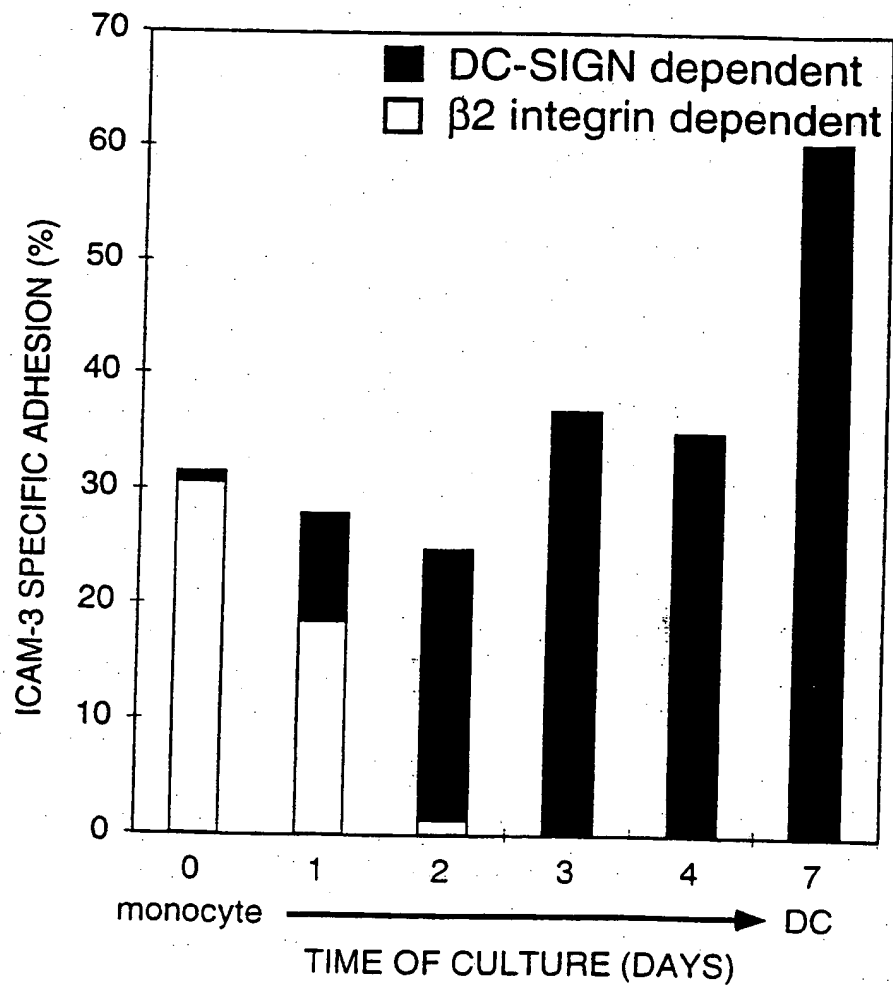
4/14

*Fig 2b*

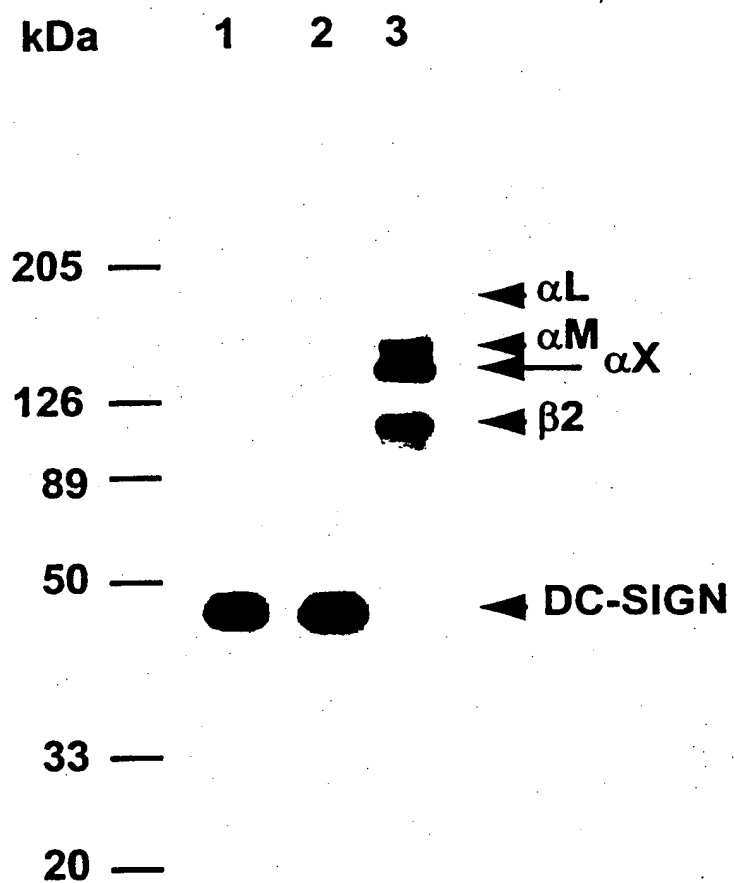
5/14



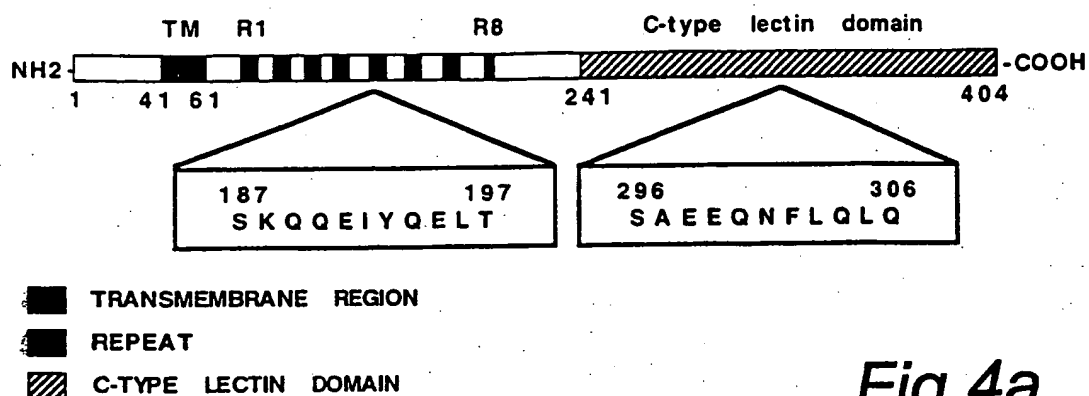
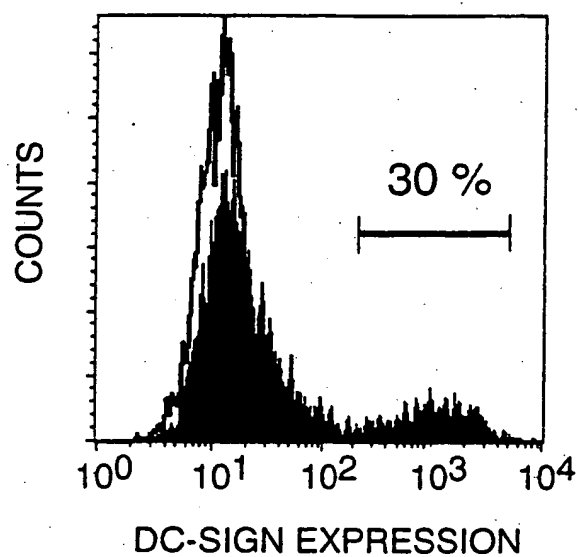
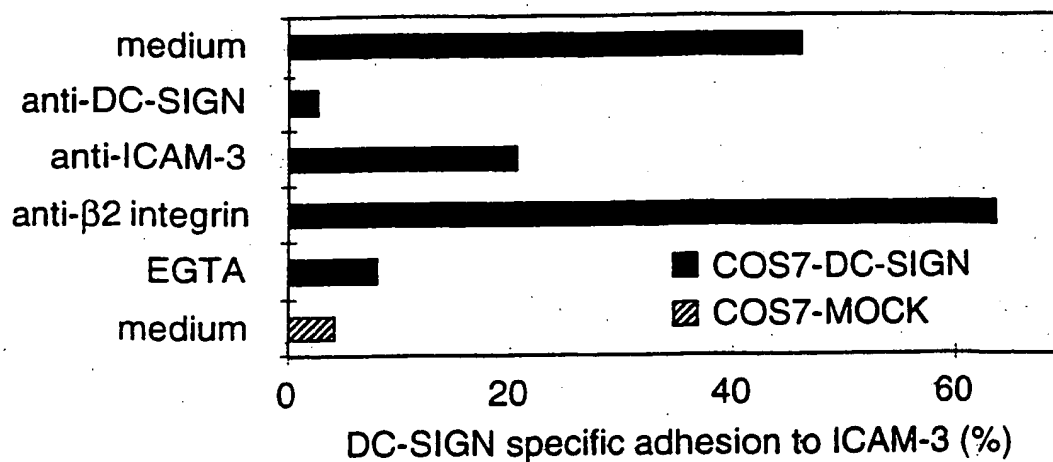
6/14

*Fig 2d*

7/14

*Fig 3a*

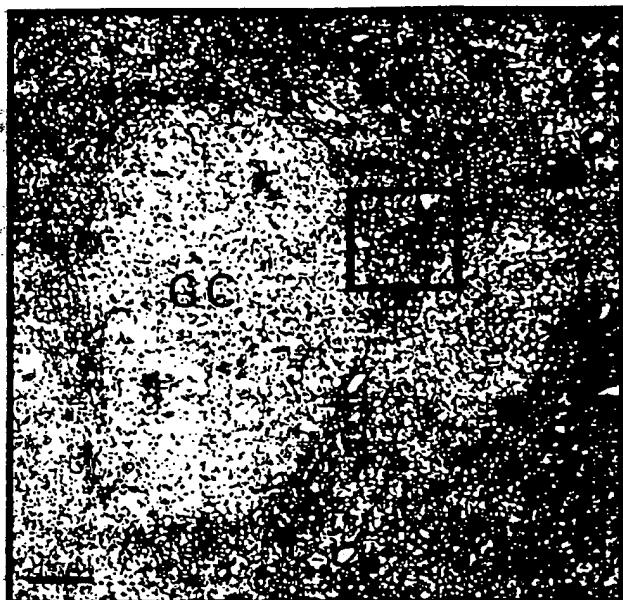
8/14

*Fig 3b**Fig 4a**Fig 4b*

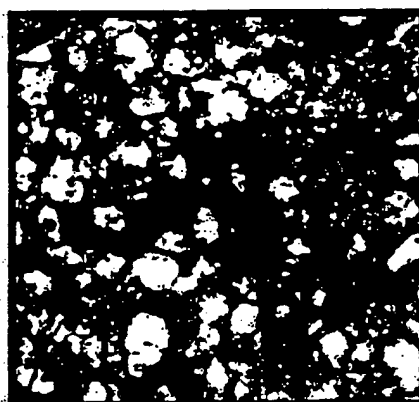
9/14

*Fig 5*

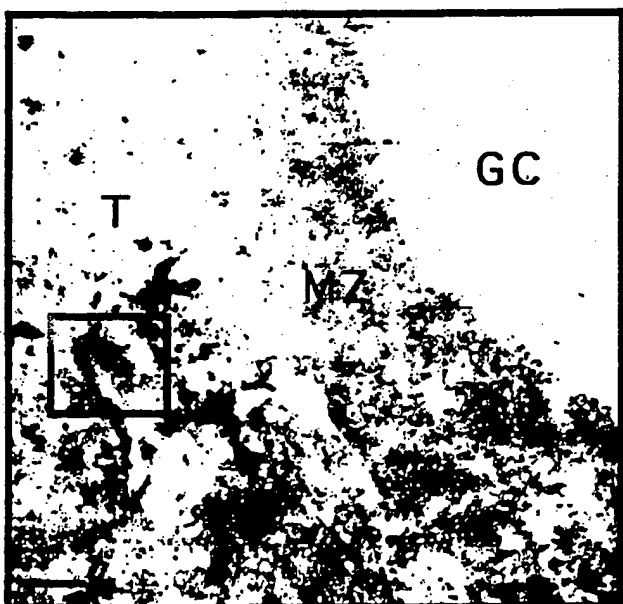
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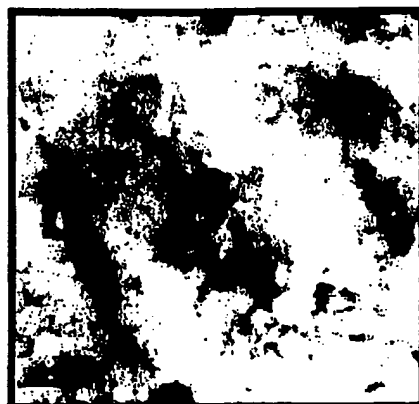
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C

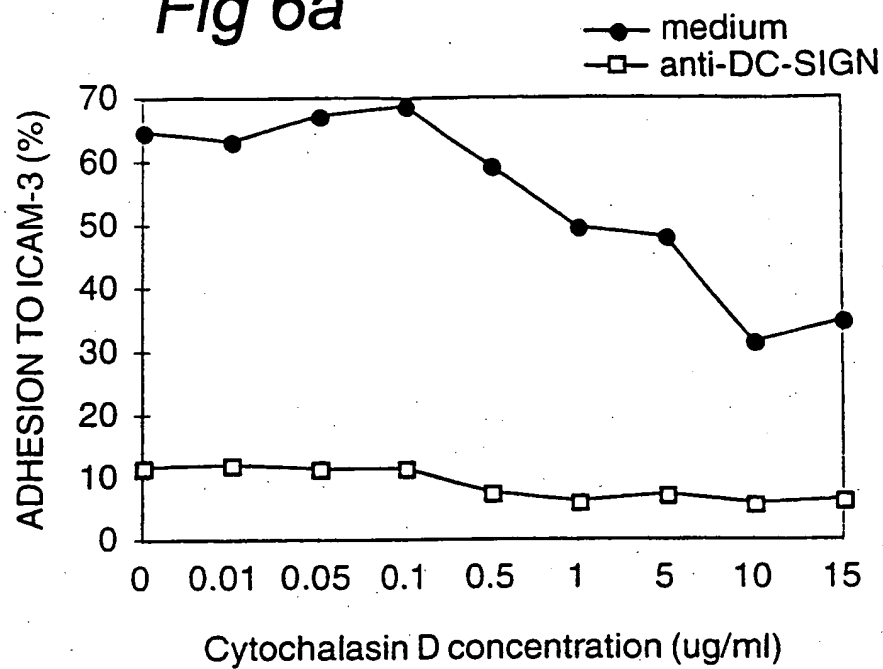
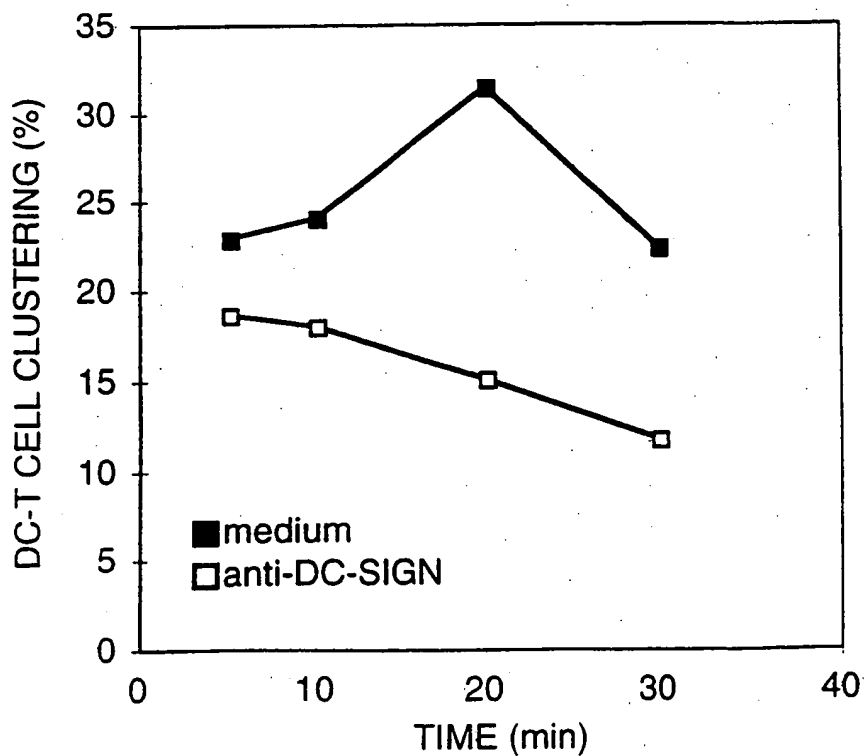


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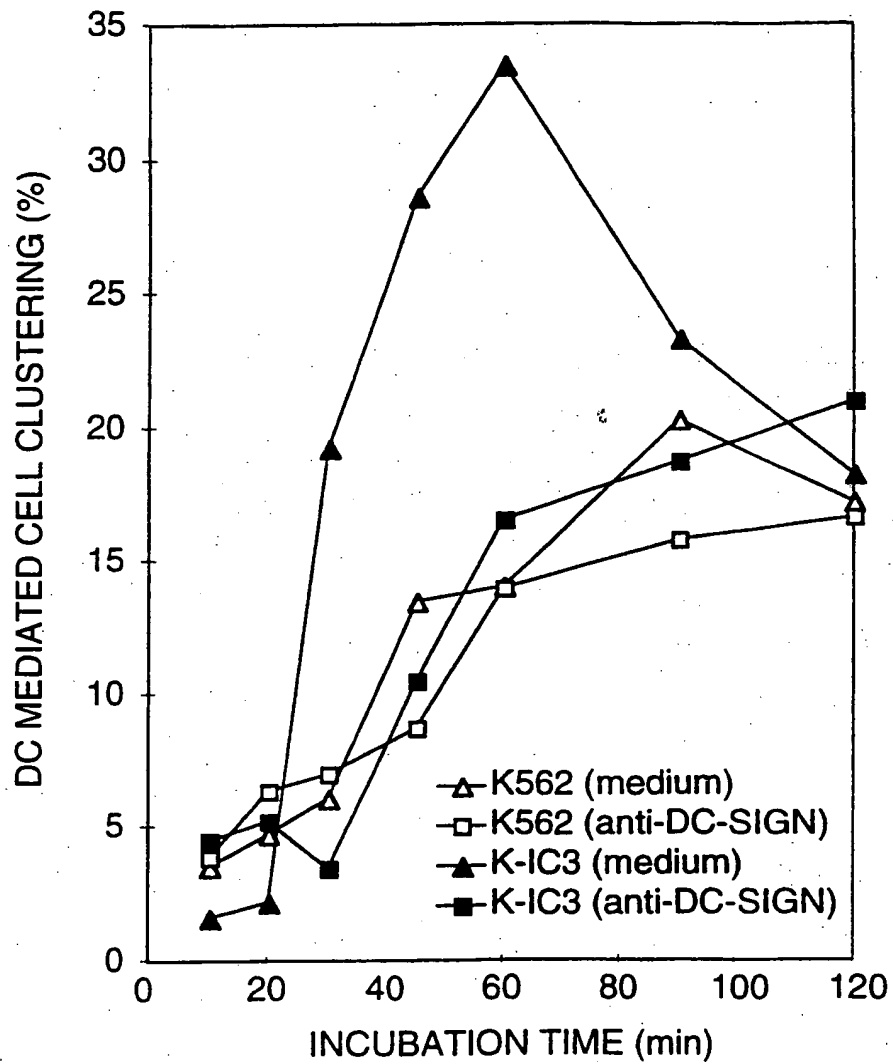


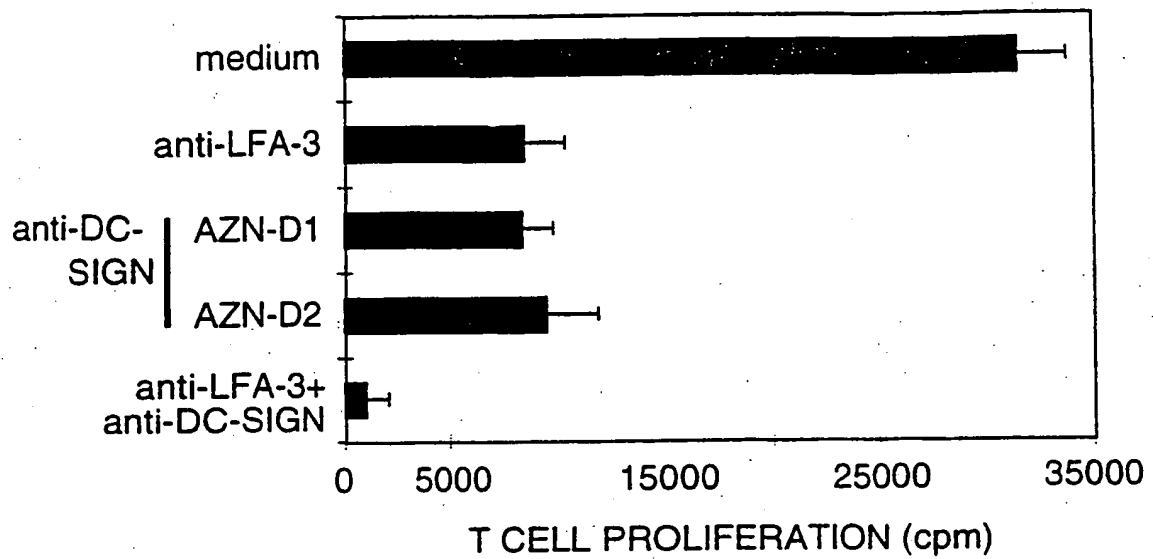
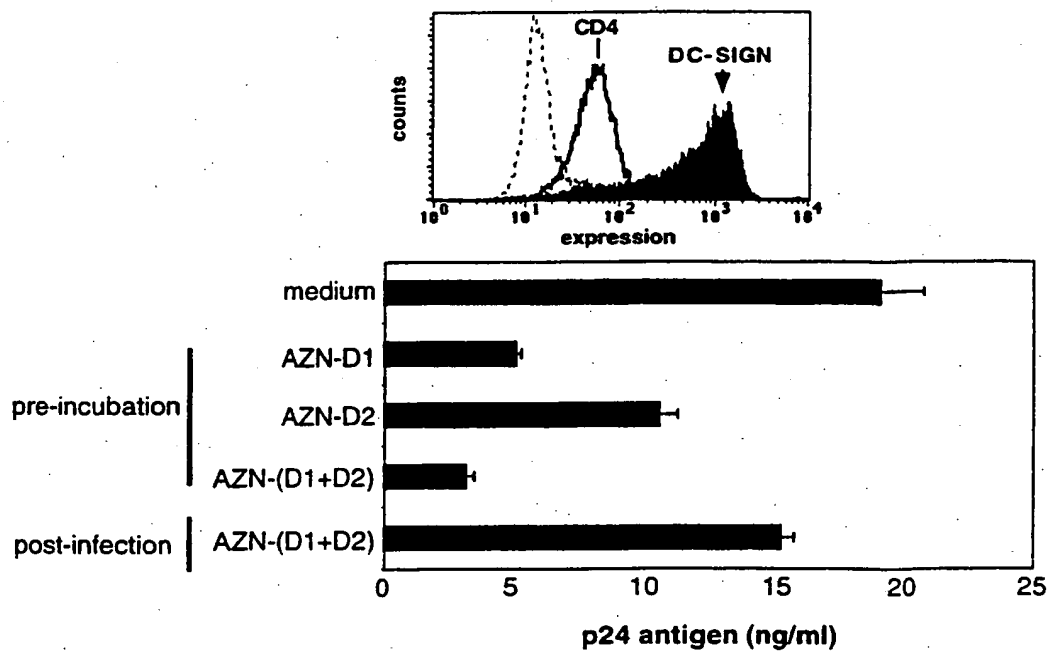
**Fig 6a**

10/14

**Fig 6c**

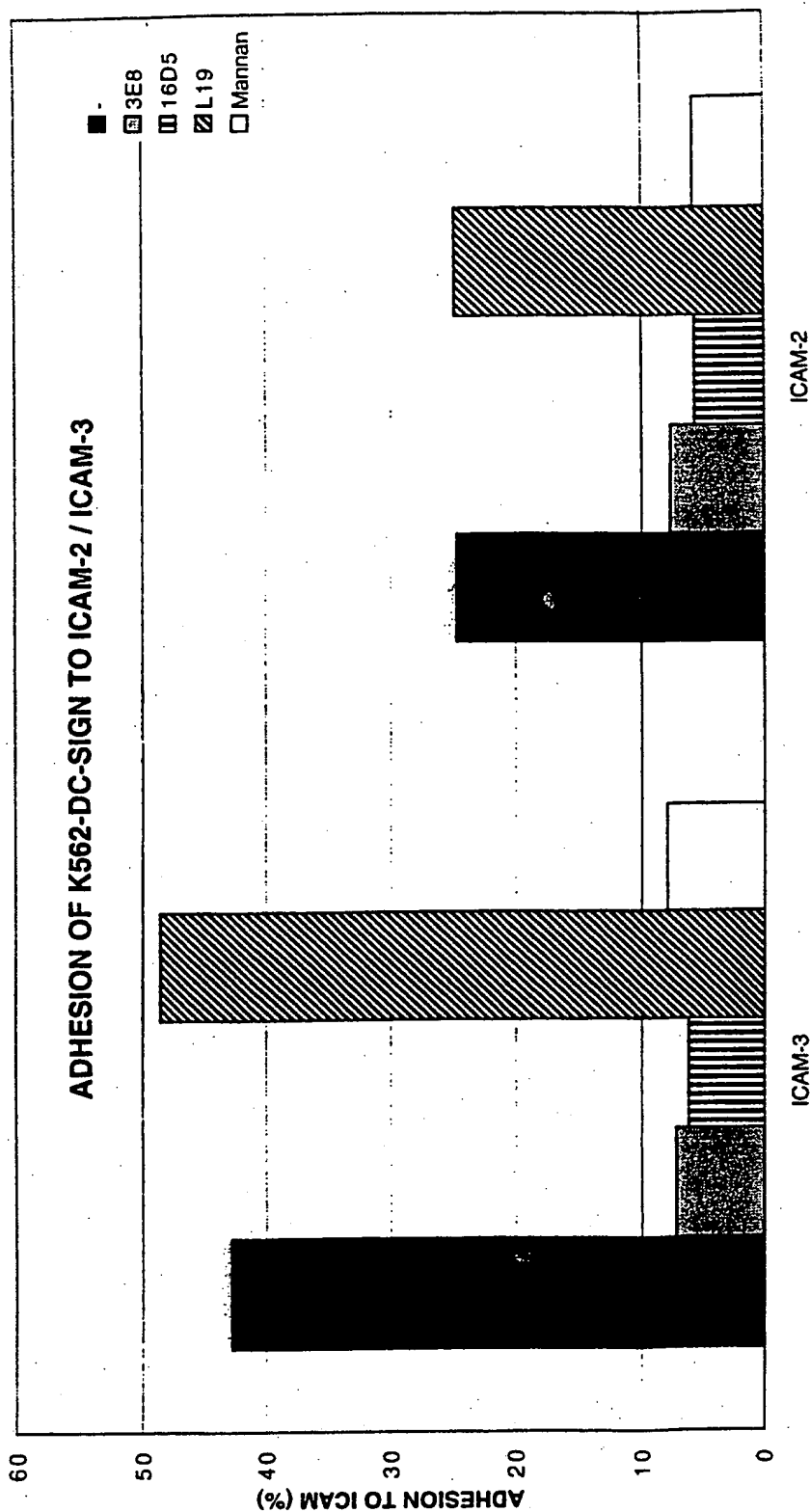
11/14

*Fig 6b*

*Fig 6d**Fig 7*

13/14

Fig 8



14/14

## Fig 9

```

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121/41
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gly pro leu val leu gln leu leu ser phe
181/61
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ser lys val pro ser ser ile ser gln glu
241/81
CTG ACC CAG CTT AAA GCT GCA GTG GGT GAG
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301/101
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361/121
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gln glu ile tyr gln glu leu thr arg leu
421/141
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481/161
CCA GAG AAA TCT AAG ATG CAG GAG ATC TAC
pro glu lys ser lys met gln glu ile tyr
541/181
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601/201
GCT GCA GTG GGT GAG CTT CCA GAG AAA TCT
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661/221
CGG CTG AAG GCT GCA GTG GGT GAG CTT CCA
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961/321
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1021/341
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331/111
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1171/391
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phe leu ser pro ala pro ala thr pro asn

```

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

(A) NAME: Koninklijke Universiteit Nijmegen,  
Afdeling tumorimmunologie  
(B) STREET: Philips van Leydenlaan 25  
(C) CITY: Nijmegen  
(E) COUNTRY: Netherlands  
(F) POSTAL CODE (ZIP): 6525 EX  
(G) TELEPHONE: + 31 243 617 600  
(H) TELEFAX: + 31 243 540 339

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(ii) TITLE OF INVENTION: COMPOSITION AND METHOD FOR MODULATING  
DENDRITIC CELL-T CELL INTERACTION

(iii) NUMBER OF SEQUENCES: 2

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1215 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Phe Leu Ser Pro Ala Pro Ala Thr Pro Asn Pro Pro Pro Ala \*  
395 400 404

# INTERNATIONAL SEARCH REPORT

Intern al Application No

PCT/NL 00/00253

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K16/28 A61K39/395 C12N5/08

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

STRAND, EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 01820 A (SQUIBB BRISTOL MYERS CO) 4 February 1993 (1993-02-04) cited in the application see Figure 3A. the whole document	11-13,22
X	CURTIS B.M. ET AL.,: "Sequence and expression of a membrane-associated C-type lectin that exhibits CD4-independent binding of human immunodeficiency virus envelope glycoprotein gp 120" PROC. NATL. ACAD. SCI. U.S.A., vol. 89, 1992, page 8356-8360 XP002118177 cited in the application figure 2	11-13,22

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
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- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

18 July 2000

Date of mailing of the international search report

02/08/2000

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Authorized officer

Mateo Rosell, A.M.

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 98 55508 A (KATO SEISHI ;PROTEGENE INC (JP); SEKINE SHINGO (JP); SAGAMI CHEM R) 10 December 1998 (1998-12-10) SEQ.ID.N.39 page 3, line 12 -page 4, line 8 page 32, line 6 -page 33, line 15</p>	11,12
X	<p>DATABASE EMBL NUCLEOTIDE AND PROTEIN SEQUENCES, 6 April 1999 (1999-04-06), XP002142774 HINXTON, GB AC = AB015629. Homo sapiens mRNA for type II membrane protein similar to HIV gp120-binding C-type lectin, complete cds, clone:HP01347. abstract</p>	11,12
A	<p>WO 96 23882 A (UNIV ROCKEFELLER ;STEINMAN RALPH M (US); NUSSENZWEIG MICHEL C (US)) 8 August 1996 (1996-08-08) cited in the application page 3 -page 8; example 1</p>	1,2,4,7, 8,10, 16-18
A	<p>WO 98 28332 A (TAKASHIMA AKIRA ;UNIV TEXAS (US); ARIIZUMI KIYOSHI (US)) 2 July 1998 (1998-07-02) abstract page 3, line 9-30 page 4, line 1-11 page 13, line 13 -page 14, line 4 page 107, line 17 -page 125, line 4</p>	1,2,4
A	<p>WO 98 02456 A (INCYTE PHARMA INC (US)) 22 January 1998 (1998-01-22) cited in the application page 1, line 1 -page 2, line 18 page 13, line 16-19 page 44, line 14 -page 45, line 21</p>	1,4, 23-26
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